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**Differential impact of anabolic and  
catabolic stimuli on differentiation of young  
and old murine and human myoblasts**

**A Elbouzidi**

**MPhil      2017**

**Differential impact of anabolic and catabolic  
stimuli on differentiation of young and old  
murine and human myoblasts**

**Aisha Elbouzidi**

**A thesis submitted in partial fulfilment of  
the requirements of the Manchester  
Metropolitan University for the degree of  
Master of Philosophy**

**Faculty of Science and Engineering**

**School of Healthcare Science**

**2017**



## Abstract

Although it is important to understand the molecular mechanisms underpinning muscle wasting with aging, it is ultimately the failure of cell function that leads to all aging phenomena. Part of the cause of cellular dysfunction in old age is thought to be chronic, low grade, systemic inflammation in the face of reduced anabolic drives. Skeletal muscle and its resident stem cells provide good models of cellular and tissue failure with age. Easy access to muscle-derived stem cells provides a tool with which to assess local vs. environmental triggers of age-related cellular dysfunction. The aim of this study was to develop relevant models of age-related muscle wasting. The hypothesis was that older muscle cells would be more prone to catabolic and less prone to anabolic adaptation vs. younger muscle cells. Objectives were to compare and contrast younger and older skeletal muscle cell adaptation to anabolic (IGF-I) and catabolic (IL-6 and TNF- $\alpha$ ) stimuli.

In the murine model, and in line with expectations, IGF-I (100 ng/ml) resulted in improved fusion in younger, but reduced fusion in older myoblasts. By contrast, and unexpectedly, in human myoblasts the opposite occurred, where IGF-I (100 ng/ml) resulted in reduced fusion in younger, but increased fusion in older myoblasts. Where improved fusion was evident (regardless of model or age) with IGF-I administration, this was associated with enhanced basal fusion potential, which should therefore be considered when choosing models of study. It is currently not known what drives altered basal fusion capability and the subsequent enhanced response to IGF-I. Therefore, the two models together provide an opportunity to further investigate this finding.

When assessing the impact of catabolic cytokines on myoblast fusion, both models displayed increased negative adaptation in older vs. younger myoblasts. However, and interestingly, older murine myoblasts were more responsive to TNF- $\alpha$  and older human myoblasts to IL-6 administration. The impaired differentiation in response to the cytokines may underlie the muscle wasting evident in older age, and again provides a good model of study.

In conclusion, evidence is provided here of the development of muscle models for investigating muscle aging in vitro. Early data suggest that care should be taken when choosing the model and this should be driven by the final question being addressed e.g., the model for studying hypertrophy may not be the best model for studying atrophy.

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I would extend my thanks to the Institute for Healthcare Science where this work was carried out. Finally, I would like to thank my awesome family: my beautiful daughter, my husband and my family for their love, patience and continuous support, for understanding that I was not able to call as regularly as I would always have wanted. The MPhil is not an easy programme, however I made it through, unscathed; thanks to everyone for their encouragement.

## Author's Declaration

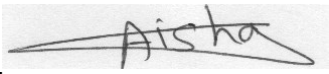
I declare that the work in this thesis was carried out in accordance with the regulations of Manchester Metropolitan University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Manchester Metropolitan University and the Institute for Biomedical Research in Human Movement and health.

This thesis has not been presented to any other university for examination either in the United Kingdom or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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## List of abbreviations

ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variances
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
°C	Celsius
Cu	Copper ion
CK	Creatine kinase
DM	Differentiation medium
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DAPI	4', 6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic acid
dNTP	Deoxyadenosine triphosphate
EGF	Endothelial growth factor
FACS	Fluorescence-activated cell sorter
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FOXO	Forkhead box subgroup O transcription factor
FSC	Forward scatter channel
g	Gram
GH	Growth hormone
GM	Growth medium

HK	Hexokinase
HS	Horse serum
Hu EGF	Human epidermal growth factor
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGFBP	Insulin-like growth factor binding protein
I-kBa	Inhibitor-kappa B alpha
IL	Interleukin
IRS-1	Insulin receptor substrate-1
IPA	Isopropyl alcohol
JAK	Janus kinase
JNK	Jun N-terminal kinase
M	Molar
MADD	MAPK activating death domain
MAPK	Mitogen-Activated Protein Kinase
MKK	Mitogen-activated protein Kinase Kinase
Myf5	Myogenic factor five
MyoD	Myogenic determination
MPCs	Skeletal muscle precursor cells
mg	Milligram
ml	Millilitre
mU	MilliUnits
MES	Mesity2-(N-Morpholino) ethane sulfonic acid
MHC	Myosin heavy chain
MRFs	Myogenic regulatory factors
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide

NADH	Nicotinamide adenine dinucleotide reduced form
NCS	Newborn calf serum
NF-Kb	Nuclear factor-kappa B
PAX3	Paired box transcription factor 3
PAX7	Paired box transcription factor 7
PAX	Paired box transcription factor
PBS	Phosphate buffered saline
6-PG	6-phosphogluconate
PI	Phosphatidylinositol or propidium iodide
PI3K	Phosphatidylinositol 3-kinases
RT	Room temperature
SD	Standard deviation
SE	Standard error
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TMT	Tris/MES Triton
TNF- $\alpha$	Tumour necrosis factor-alpha
TNFR-1	Tumour necrosis factor receptor 1
TNFR-2	Tumour necrosis factor receptor 2
TRADD	TNF receptor-associated death domain
TritonX-100	(t-otyl phoshoxyppoly-ethoxyethanol)
Tris base	Tris (hydroxymethyl) aminomethane
$\mu\text{g}$	Microgram
$\mu\text{m}$	Micrometre
$\mu\text{L}$	Microlitre

## **Chapter 1**

### **Introduction**



## **1.1 Literature review**

### **1.1.1. Skeletal Muscle**

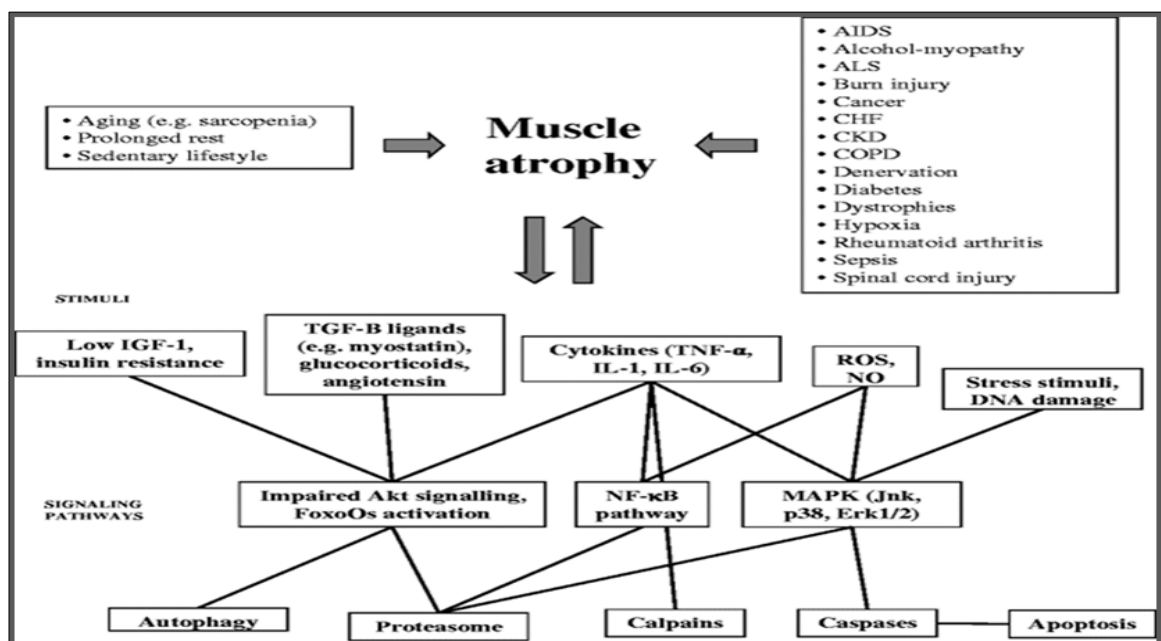
The human body is largely comprised of skeletal muscle and accounts for 50% of the total body mass in young age. This reduces about 25% of total bodyweight by 75-80 years of age (Kalyani *et al.*, 2014). It is the largest protein reservoir and is a source of amino acids utilised for energy production by a variety of organs during catabolic periods, such as starvation depletion, burn injury, heart failure, cancer, sepsis, and AIDS (Figure.1.1). In order to produce glucose, the liver uses amino acids from muscle protein breakdown (Bonaldo & Sandri, 2013; Wolfe, 2006). In addition, with aging, both muscle mass and regenerative capacity are gradually lost (Manini *et al.*, 2013).

When protein degradation exceeds protein synthesis, reduced muscle bulk occurs leading to a decrease of the cross-sectional area of myofibres and muscle strength (Schiaffino & Dyar, 2013; Kalyani *et al.*, 2014). Moreover, extreme protein degradation in skeletal muscle has severe consequences on the human body including the possibility of death (Bonaldo & Sandri, 2013; Argilés *et al.*, 2016). Furthermore, the effectiveness of various therapeutic treatments can be impaired by the disproportionate loss of muscle mass (Wüst & Degens, 2007; English & Douglas Paddon-Jones, 2010; Kalyani *et al.*, 2014). The prevention of metabolic disorders, facilitation of healthy age progression and the provision of energy to vital organs during stressed conditions largely depends on the maintenance of healthy muscles. In adults, muscle mass and performance adjust to diverse pathophysiological conditions through the activation of pathways that control protein turnover (Bonaldo & Sandri, 2013). Endurance, strength, and physical performance are largely contingent upon skeletal muscle mass (Costa *et al.*, 2012).

### **1.1.2. Skeletal muscle atrophy**

During atrophy, there is a major reduction in protein content, fibre diameter, force production, and fatigue resistance (Dodson *et al.*, 2011; Williamson *et al.*, 2003). It is a typical response to such conditions as starvation, aging (sarcopenia) and inactivity. However, it may also present as a symptom of other chronic diseases such as cancer (cachexia), diabetes, sepsis, AIDS, renal and heart failure (Fanzani *et al.*, 2012). These all contribute to the morbidity and early mortality of the patient (Vinciguerra *et al.*, 2010).

Many triggers of significant decreases in muscle, body weight and fat are due to increased protein catabolism driven by tumour-derived factors, anorexia and endocrine changes (Ebadi & Mazurak, 2014). Important endocrine changes include elevated TNF- $\alpha$ , which is known to induce skeletal muscle catabolism via, among others, the ubiquitin-proteasome system (UPS) (Sandri, 2013; Bonaldo & Sandri, 2013). Increased catabolic signalling, oxidative stress, reduced anabolic signalling, and reduced protein synthesis are additional processes involved in the pathogenesis of muscle atrophy (McCarthy & Esser, 2010; Bonaldo & Sandri, 2013).



**Figure 1.1. Muscle atrophy.**

Muscle atrophy can result as a consequence of many different physiological and pathological conditions. (Fanzani *et al.*, 2012).

### 1.1.3. Sarcopenia

Age-related muscle loss (termed sarcopenia) is the loss of skeletal muscle mass and function associated with aging. It is a serious geriatric clinical disorder affecting more than half of the population over 80 years (Cruz-Jentoft *et al.*, 2010; Loeser, 2010). Humans over the age of 50 generally lose 1-2% of muscle mass per year (Hughes *et al.*, 2002). This has a direct effect on muscle strength, muscle functionality, and metabolic performance and ultimately morbidity and mortality (Bonaldo & Sandri, 2012; Muscaritoli *et al.*, 2013). Sarcopenia is multifactorial and includes factors such as chronic inflammatory diseases, disuse and malnutrition (Biolo *et al.*, 2014).

The decline in muscle mass with aging is accompanied by a decrease in the number and size of muscle fibres (Michel *et al.*, 2004; Evans, 2010) . It has been established that by the eighth decade a 50% decline in the total fibre number is present when compared with muscle tissue in young subjects and that the cross- sectional area of type II fibres is nearly 35% smaller in muscles from older individuals when compared with young individuals (Mitchell *et al.*, 2012; Nilwik *et al.*, 2013). A growing body of literature generally agrees that type II fibre atrophy occurs with age whereas type I fibre size remains unchanged

(Deschenes, 2004; Lee *et al.*, 2006). Researchers have demonstrated that any differences detected could either be attributed to age or simply inactivity (Klitgaard *et al.*, 1990). Studies have been conducted that show that elderly strength-trained subjects have cross-sectional areas larger than that of sedentary subjects and similar to those of young adult control subjects, proposing that age-related changes in skeletal muscle function may be responded to by long-term strength-training in elderly men.

Increased levels of circulating cytokines and catabolic hormones are associated with muscle atrophy (Figure.1.1) (Costamagna *et al.*, 2015b), however, the current lack of available effective therapies to slow or reverse muscle loss is hampered by limitations in the understanding of the pathogenesis of muscle atrophy (Bodine, 2013).

#### **1.1.4. The role of satellite cells in adult muscle**

The impaired function of skeletal muscle stem cells or satellite cells may be a factor underlying muscle atrophy (Mann *et al.*, 2011; Snijders *et al.*, 2015). Satellite cells are mono-nucleated myogenic precursor cells (myoblasts) located between the basal lamina and the plasma membrane of muscle fibres and are primary factors in muscle regeneration (Yablonka-Reuveni, 2011; Yin *et al.*, 2013). While they are generally in a quiescent undifferentiated state, when activated in reaction to muscle damage, they are able to proliferate, migrate to the site of injury and fuse with existing fibres for regeneration and hypertrophy (Mauro, 1961; Yin *et al.*, 2013; Dumont *et al.*, 2015).

### 1.1.5. Impairment of satellite cell function in repair and hypertrophy

The regenerative capacity of postnatal skeletal muscle is deemed largely dependent on satellite cells (Figure.1.2).

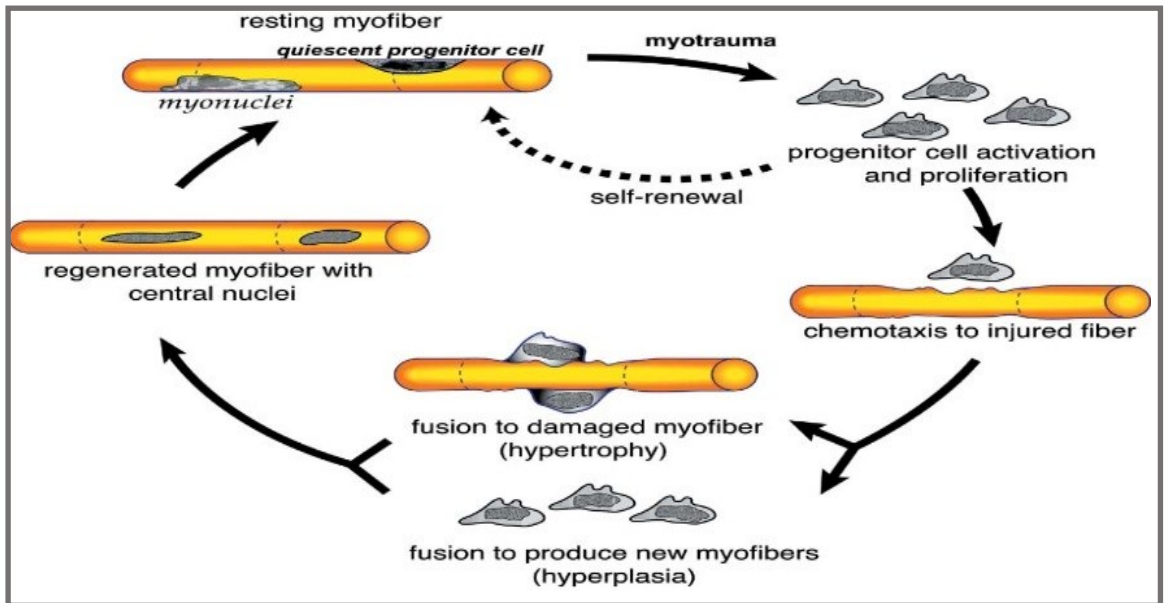


Figure 1.2. Role of satellite cells in muscle regeneration (Hawke and Garry, 2001).

During embryogenesis, skeletal muscle precursor cells (MPCs) originate in somites and undergo a multi-process of lineage commitment, migration, proliferation and differentiation (Kawiak *et al.*, 2006; Bentzinger *et al.*, 2012) (Figure.1.3).

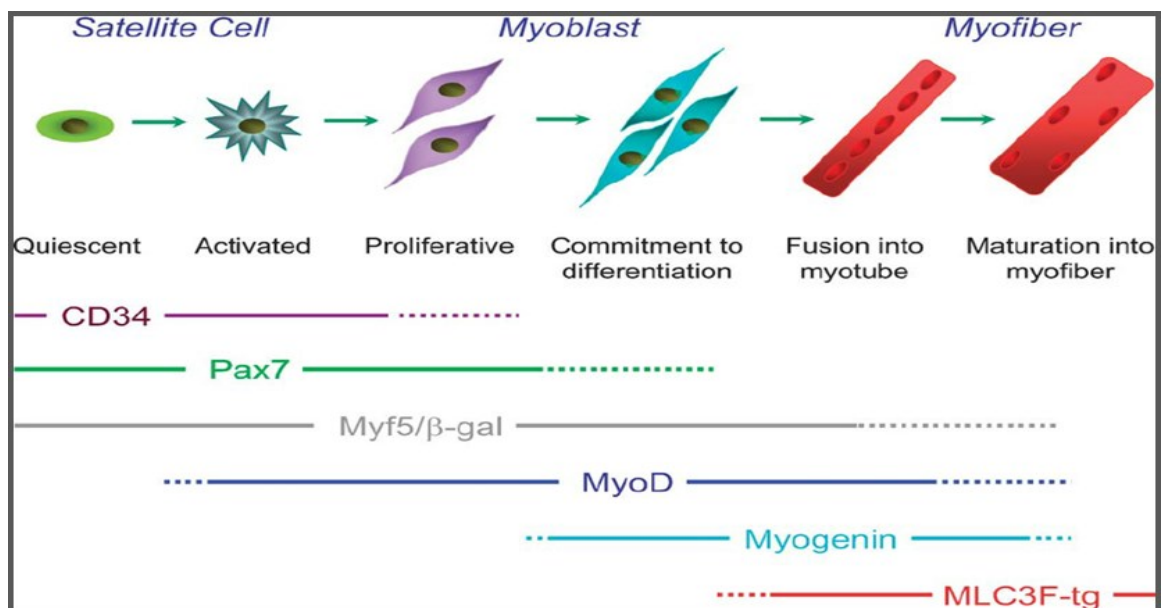


Figure 1.3. Satellite cell activation and maturation. Adapted from (Zammit *et al.*, 2006).

#### **1.1.6. Age-dependent decline in stem cell functionality of satellite cells**

The decrease in the stem cell functionality of satellite cells with age, is possibly attributed to intrinsic molecular variations in aged muscle stem cells or extrinsic changes due to the aging of the local microenvironment (*e.g.* myofibres and the extracellular matrix) or the systemic environment (Dumont *et al.*, 2015). Several groups have reported age-associated reductions in the number of satellite cells and hypothesised that this loss of satellite cells ultimately contributes to the aged-related muscle atrophy (Shadrach & Wagers, 2011; Oh, 2015; Snijders *et al.*, 2015).

The murine C2 and C2C12 skeletal myoblasts provide established models to investigate some of the processes of muscle cell adaptation during aging. Historically, research within our laboratory has been conducted using C2 cells that were originally established following C3H leg muscle crush-injury (Yaffe & Saxel, 1977). The daughter C2C12 cells are a subclone of the parental C2 line (Chiu & Blau, 1985) and were generated for their differentiation potential. The former provides a model for younger muscle cells with sustained hypertrophy potential, while the latter provides a model of atrophying older muscle (Sharples *et al.*, 2010). Use of the two lines in parallel, provide an opportunity to begin to investigate cellular adaptation with age and inflammation.

#### **1.1.7. Skeletal muscle hypertrophy**

Skeletal muscle hypertrophy is defined by an increase in the muscle mass and in the cross-sectional area of the fibres (Paul & Rosenthal, 2002). Generally, muscle hypertrophy is related to strength training or conditions associated with increased protein synthesis (Schoenfeld, 2010; Kalyani *et al.*, 2014).

The induction of hypertrophy is associated with incorporation of new myonuclei (provided by satellite cells) into the fibres which enable increased protein synthesis and a constant myonuclear domain (Shenkman *et al.*, 2010). During the embryonic and post-embryonic periods, the satellite cells play an important role in muscle growth. It is accepted that satellite cells have an important role during muscle hypertrophy, also in adults, given appropriate stimuli. Some controversy does, however, exist, as McCarthy *et al* (2011), using a genetic mouse model to conditionally ablate satellite cells, reported that while satellite cells were not required for muscle hypertrophy, they were essential for the formation of

new fibres and for muscle regeneration (McCarthy *et al.*, 2011). Therefore, although the precise role of the satellite cell in adult hypertrophy is debatable, there is a clear need for these cells in regeneration.

#### **1.1.8 Insulin-Like Growth Factor (IGF-I)**

As mature muscle fibres are post mitotic, in order to enable regeneration, they are dependent on the recruitment of satellite cells, which involves both cytokines and growth factors. An important growth factor in skeletal muscle biology is Insulin like Growth Factor I (IGF-I) (Shadrach & Wagers, 2011; Philippou *et al.*, 2007). Indeed, IGF-I has been shown to enhance protein synthesis, to increase the size of myotubes, (Velloso, 2008), to suppress protein degradation (Wang & Mitch, 2014), and to increase the number of satellite cells (Velloso, 2008; Zhang *et al.*, 2010). Indeed, increased endogenous expression of the IGFs in murine myoblast cell lines (e.g. C2 and C2C12) is associated with spontaneous fusion upon transfer to low serum differentiation medium (Florini *et al.*, 1991; Stewart & Rotwein, 1996).

The IGF system includes: two IGFs (IGF-I and IGF-II), the type I and type II IGF cell surface receptors, six specific high-affinity binding proteins (IGFBPs-1 to -6), IGFBP proteases, and other IGFBP-interacting molecules (Clemmons, 1997). All play a role in embryonic, foetal and adult development and perform a wide array of biological functions (Sharples *et al.*, 2015). Reduced levels of the IGF-I *gene* transcripts in skeletal muscle (by up to 45% in older vs. younger males (Léger *et al.*, 2008) during aging (Benbassat *et al.*, 1997; C. Velloso, 2008), may be associated with a reduction in Growth Hormone (GH) levels and sensitivity (Figure.1.4).

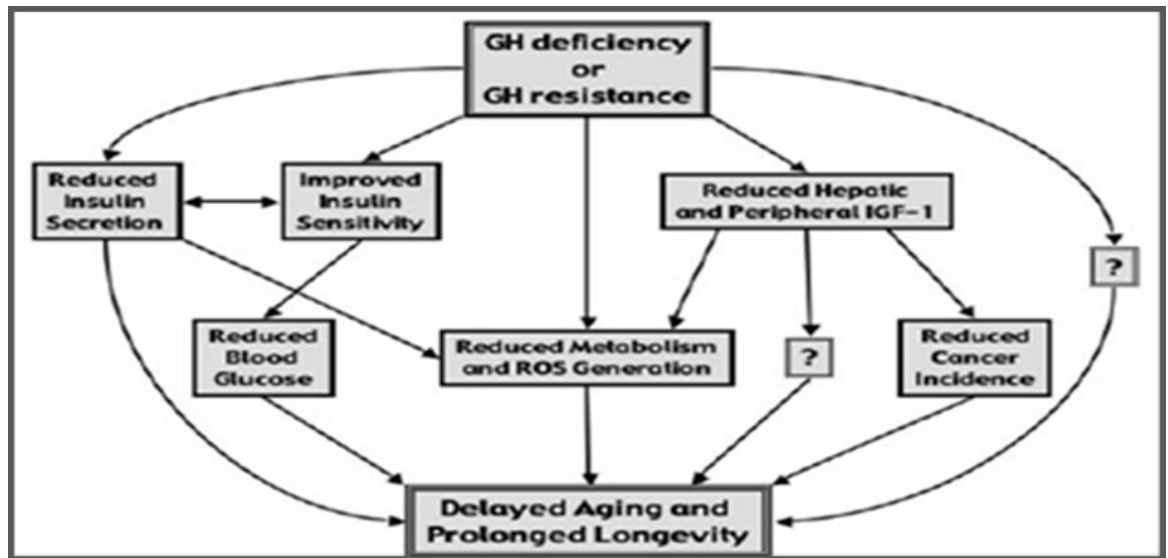


Figure 1.4. GH deficiency and Aging (Bartke, 2005).

The binding of IGF-I to its receptor stimulates several downstream signalling events including the activation of the MAP kinase (Erk 1/2) and PI 3-kinase pathways as detailed in Figure.1.5 (Singleton & Feldman, 2001).

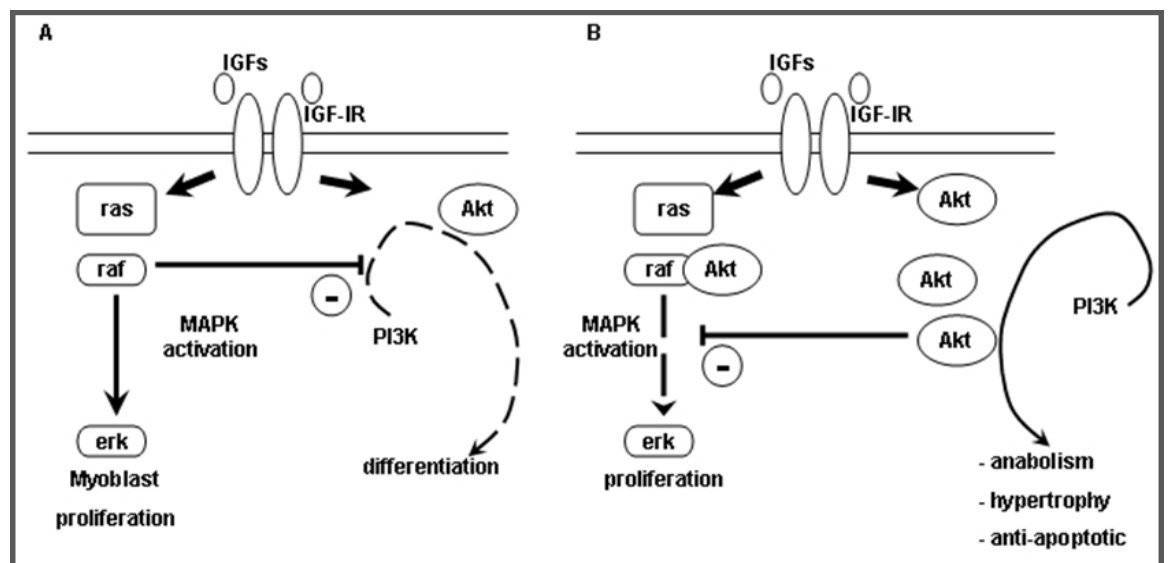


Figure 1.5. The role of IGF in stimulating proliferation and differentiation (Singleton and Feldman et al 2001).

In human studies, muscle from older men has higher levels of MAPK proteins compared to those of younger men (Williamson *et al.*, 2003). The importance of the PI3K/Akt pathway has been illustrated in C2C12 myoblasts using inhibitors of PI3K, which attenuated the process of hypertrophy (Rommel *et al.*, 1999; Glass, 2005; Sharples & Stewart, 2011). Substantiation has been provided in animal models through the knock down of Akt1, which resulted in smaller and lighter animals compared with wild type control (Florian Bentzinger *et al.*, 2013). Furthermore, work by (Lai *et al.*, 2004) demonstrated that activation of Akt was sufficient to cause a 73% increase in quadriceps muscle size in mice. Thus the age-related decrease in IGF-I may underlie the decreased proliferation and differentiation of myoblasts in older age (Arthur & Cooley, 2012). Furthermore, IGF-I has been shown to decrease fibrosis and control the inflammatory response following injury, by down-regulating pro-inflammatory cytokines (Specia *et al.*, 2012).

#### **1.1.9 Tumour Necrosis Factor Alpha (TNF- $\alpha$ )**

TNF- $\alpha$ , a pleiotropic pro-inflammatory cytokine, which along with other cytokines is secreted by a number of cells such as: myoblasts, neutrophils, macrophages, fibroblasts, vascular smooth muscle cells and vascular endothelium (Li & Reid, 2000; Otis *et al.*, 2014). These cytokines are usually produced in response to damage or infection of tissue and form part of the normal inflammatory response (Zhang & Zheng, 2007). However, in diseases such as advanced HIV, cancer, chronic infections (Deeks *et al.*, 2013; Ali *et al.*, 2013) and sarcopenia (Sakuma & Yamaguchi, 2012), prolonged and increased expression of these cytokines are believed to underpin catabolism and therefore wasting of skeletal muscle (Grounds, 1998; Reid & Li, 2001; Tajrishi *et al.*, 2014). TNF- $\alpha$  blocks myogenic differentiation (Li *et al.*, 1998; Langen *et al.*, 2001; Li *et al.*, 2014) and causes extensive cell death in C2 cells (Stewart *et al.*, 2004) via a plethora of signalling pathways (Figure.1.6). Cell culture studies have additionally demonstrated that prolonged exposure of differentiated myotubes to clinically relevant levels of TNF- $\alpha$  (1-6 ng/ml) stimulates muscle protein degradation (Li *et al.*, 1998).



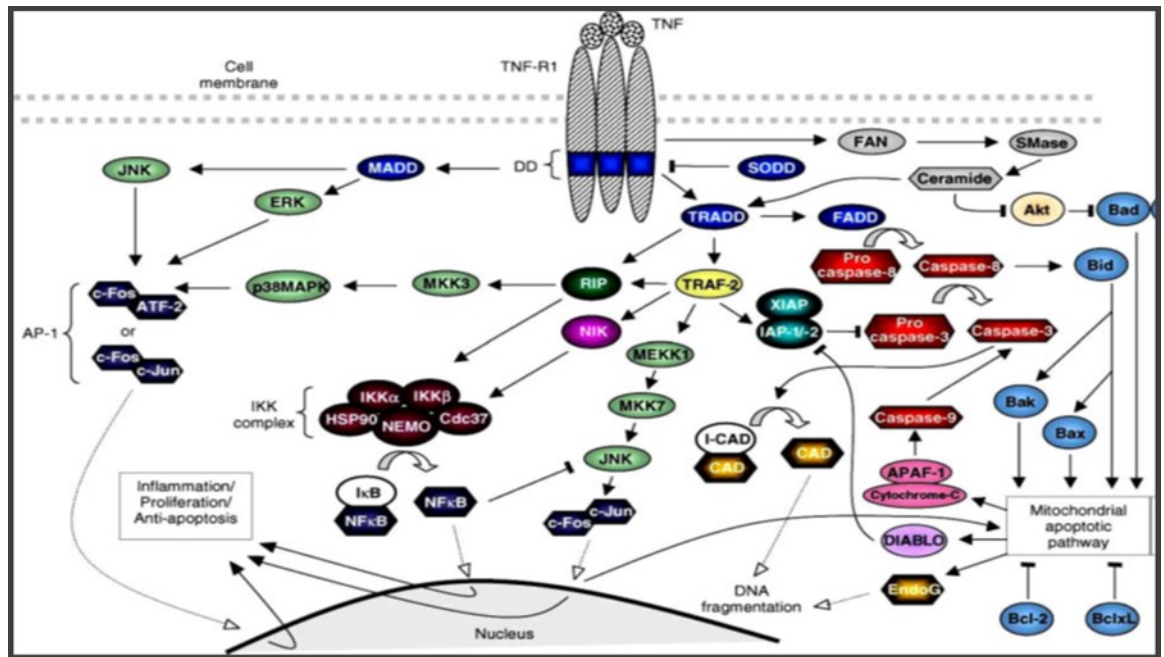


Figure 1.6. TNF- $\alpha$  signalling pathway. Taken from (Mocellin et al., 2005).

### 1.1.10 Interleukin (IL-6)

IL-6, a pleiotropic cytokine, is produced by a number of cells including: myoblasts, myofibres, adipocytes, macrophages, neutrophils, endothelial cells and osteoblasts (Yin *et al.*, 2013; Muñoz-Cánoves *et al.*, 2013). IL-6 exerts its effects following cell surface receptor binding and activation of a host of signaling pathways, illustrated in Figure.1.7).

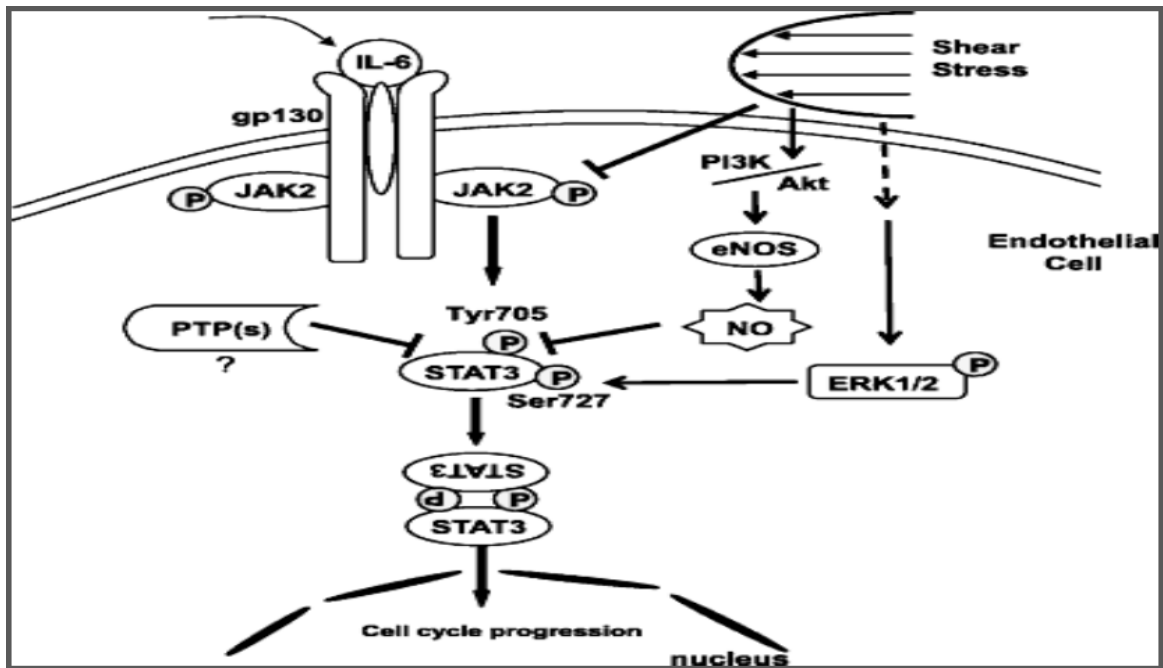


Figure 1.7. IL-6 signalling pathway. Taken from (Ni *et al.*, 2004).

IL-6 mRNA knockdown decreases muscle-specific gene expression in cultured C2C12 myoblasts (Baeza-Raja & Muñoz-Cánoves, 2004; Serrano *et al.*, 2008), suggesting an anti-myogenic role for this cytokine (Muñoz-Cánoves *et al.*, 2013). IL-6 infusion or over-expression in murine models induces muscle atrophy (Muñoz-Cánoves *et al.*, 2013; B. K. Pedersen & Steensberg, 2004). Despite these data, IL-6 is also reported to have beneficial effects in skeletal muscle, being linked with hypertrophy and myogenesis (Serrano *et al.*, 2008; Muñoz-Cánoves *et al.*, 2013). Supportive evidence derived from IL-6 knock-out models revealed a blunted hypertrophy response compared with wild-type controls. The contrasting effects of IL-6 on muscle remain to be explained, but one possibility is that endogenous levels produced during non-inflammatory situations are beneficial, whereas when IL-6 is overproduced in scenarios where inflammation or other pathological processes are occurring it is detrimental.

#### **1.1.11 Protein synthesis/degradation imbalance**

An imbalance between protein synthesis and protein degradation, resulting in the increase of damaged proteins, is also associated with aging skeletal muscle and sarcopenia ( Kalyani *et al.*, 2014). The rate of protein synthesis in aged muscle is diminished. There are numerous causes of decreased protein synthesis in aged skeletal muscle, such as underproduction of circulating and tissue-associated GH and IGF-I (Arthur & Cooley, 2012). Low levels of IGF-I in aged muscle are related to the high expression of muscle growth inhibitors including the pro-inflammatory cytokines TNF- $\alpha$  and IL-6. In skeletal muscle of aged (70 y) subjects, there is a 45% decrease in growth hormone receptor protein (GHRS) and IGF-I mRNA, along with a 2.8 fold increase in TNF- $\alpha$  mRNA relative to young (20 y) subjects (Arthur & Cooley, 2012). Aged women with lower IGF-I levels and greater IL-6 relative to younger women, demonstrate aspects of disability and early mortality (Arthur & Cooley, 2012).

## 1.2. Summary

Although it is important to understand the molecular mechanisms underpinning muscle wasting with aging, it is ultimately the failure of cell function that leads to all aging phenomena. Part of the cause of cellular dysfunction in old age is thought to be chronic, low grade, systemic inflammation in the face of reduced anabolic drives. Skeletal muscle and its resident stem cells provide good models of cellular and tissue failure with age. Easy access to muscle-derived stem cells provides a tool with which to assess local vs. environmental triggers of age-related cellular dysfunction.

At the outset of this programme of research, it was hypothesised that murine and human myoblasts could be utilised interchangeably as models of muscle aging and atrophy. To investigate this concept, in **chapter 3**, models of older (C2) and younger (C2C12) murine myoblast lines are used. The impact of anabolic (IGF-I) and catabolic (IL-6 and TNF- $\alpha$ ) stimuli on their differentiation capacity are investigated. **Chapter 4** extends these studies through the use of primary myoblasts derived from old and young human muscle. These cells are similarly exposed to anabolic (IGF-I) and catabolic (IL-6 and TNF- $\alpha$ ) stimuli, with differentiation as the output measure. The objectives were to investigate the basal fusion potential of older and younger myoblasts and to examine the subsequent impact of growth factor/cytokine administration on myoblasts differentiation. Finally, in **chapter 5**, the potential of these cells as future models of aging muscle are compared and contrasted.

## **Chapter 2**

### **Materials and Methods**

## 2.1. Materials

### 2.1.1. Chemicals, solvents and reagents

Details regarding plastic ware and chemicals and solvents (analytical (AnalaR) or molecular biology/tissue culture grade) are presented in Tables.2.1 and 2.2.

**Table 2.1. List of Plastic ware**

<b>Name</b>	<b>Company</b>
Plastic ware	Greiner Bio-one, (Kremsmunster, Austria)
Cryogenic vials	Nalgene, (NY, USA),
Cell scrapers	Nalgene, (NY, USA),
Syringes	Terumo, (Leuven, Belgium)
500-ml bottle top filters	Corning, (NY, U.S.A)
0.22 and 0.2 $\mu$ m syringe filters	Corning, (NY, U.S.A)
96-well clear UV plates	BD Biosciences (San Jose, USA)
96-well plates for protein assays	Thermo Fisher Scientific ( Roskilde, Denmark)
Flowcytometry tubes	BD Biosciences (San Jose, USA)
Pipette tips for tissue culture	Fisher Scientific UK (Loughborough, UK)
Neubauer haemocytometer	(Sondheim, Germany)
Isopropyl alcohol (IPA) freezing chamber	Nalgene (NY, USA)

**Table 2.2. List of tissue culture reagents**

<b>Name</b>	<b>Company</b>
Heat-inactivated (hi) foetal bovine serum (FBS)	(Life Technologies,UK )
Heat-inactivated newborn calf serum (hiNCS)	Gibco (Paisley, Scotland)
Heat-inactivated horse serum (HS)	TCS Biosciences (Corby, England)
Penicillin/streptomycin and trypsin	Bio Whittaker (Wokingham, England)
L-glutamine	BDH (Poole, England)
Gelatin type A from porcine skin	Sigma (St. Louis, USA)
Glass ware	Oxoid (Basingstoke, England)
Phosphate-buffered saline (PBS)	Oxoid (Basingstoke, England)
Trypsin or TryPLE Express	(Life Technologies, UK)
Medium 199	(Invitrogen, UK)
Gentamycin	(Invitrogen.UK)
Recombinant human epidermal	(Sigma, UK)
growth (Hu EGF) and dexamethasone	(Life Technologies, UK)
Recombinant human insulin	(Life Technologies, UK)
Epidermal growth factor (EGF)	(Life Technologies, UK)
Recombinant human fibroblast	(Life Technologies, UK)
growth factor-basic (FGFb)	(Life Technologies, UK)
Recombinant human fibroblast growth-basic (FGFB)	(Life Technologies, UK)
Bovine fetuin	(Life Technologies, UK)
Dimethyl sulphoxide ( DMSO)	(BDH, UK)
Trypan blue stain	(Bio Whittaker, UK)
Texas Red_-X Phalloidin	(Sigma Aldrich, UK)
Anti-Myosin Heavy Chain (MyHC)	(Bioscience, UK)
DAPI (4',6-diamidino-2-phenylindole)	(IHC World, USA)

### **2.1.2. Biochemical assays**

Protein and creatine kinase (CK) activity analyses were performed using a Bio-Tek ELISA plate reader and analysed with Bio-Tek KC Junior Microplate. Data Analysis Software, (Winooski, USA). Reagents for protein assays were purchased from Pierce (Division of Thermo Fisher Scientific, Roskilde, Denmark) and CK reagents were from (Catachem, USA).

### **2.1.3. Cell Origin**

Mouse C2 skeletal myoblasts were originally from the American Tissue Culture Collection (ATCC) Rockville, MD, USA. They are derived from crush-injured murine C3H leg muscle (Yaffe & Saxel, 1977). Human cells were kindly donated by the laboratories of Prof. G. Butler-Browne. The compositions of cell culture media are presented in table. 2.3.

#### 2.1.4. Cell culture medium

Table 2.3. Composition of cell culture media

Types of medium	Mouse cells	Human cells
<b>Growth medium (GM)</b>	GM constituted: 400 ml DMEM with foetal bovine serum (hiFBS; 10%), hi newborn calf serum (hiNCS; 10%), L-glutamine (2 mM), and penicillin-streptomycin. (1%). GM was stored at 4°C and used within 2 months.	GM constituted: 320 ml DMEM and 80 ml Medium199. Final concentrations of supplements were as follows: hiFBS (20%), gentamycin (50 µg/ml), dexamethasone (0.2 µg/ml) recombinant human epidermal growth (Hu EGF; 5 ng/ml), bovine fetuin (25 µg/ml) recombinant human fibroblast growth-basic (rhFGF; 0.5 ng/ml) and recombinant human insulin (5 µg/ml). GM was stored at 4°C and used within 2 months.
<b>Differentiation medium (DM)</b>	DM constituted: DMEM, hiHS (2%), Pen-strep (penicillin/Streptomycin) (1%), L-glutamine (2 mM). DM was stored at 4°C and used within 2 months.	DM constituted DMEM, insulin (10 µg/ ml), gentamycin (50 µg/ ml). DM was stored at 4°C and used within 2 months.



### **2.1.5. Cell Handling**

All cell culture was performed in a microbiological safety cabinet (Labcaire SC-R Recirculating Class II, UK). All cells were incubated in a Triple Red Laboratory Technology Nuair<sup>TM</sup> DH AutoFlow CO<sub>2</sub> Air Jacketed Incubator (Buckinghamshire, UK). A Leica DMI6000B microscope (Wetzlar, Germany) was used to capture photomicrographs.

## **2.2. Methods**

### **2.2.1. Culture of skeletal myoblasts**

The C2C12 cells are a subclone of the C2 cell line (Chiu & Blau, 1985). Human C83 cells are from an older donor and C25 from a younger donor. Murine and human myoblasts maintain a fibroblast-like phenotype in GM, but rapidly withdraw from the cell cycle and undergo myogenic differentiation upon serum reduction (transfer to DM). Growth of cell cultures to confluency and routine maintenance were carried out in a class II laminar flow hood using aseptic techniques. Cells were rapidly thawed and seeded onto 0.2% gelatine-coated 75-cm<sup>2</sup> flasks supplemented with GM. Cells were maintained in humidified incubators at 5% CO<sub>2</sub> and 37°C. Media were replaced every 24-48 hours. Upon attaining 90-100% confluency cells, were washed in phosphate-buffered saline (PBS), trypsinised, counted and sub-cultured for expansion or experimentation. Cell viability was determined using trypan blue counting and a Neubauer haemocytometer (see below and Figure.2.1). Cells not used for experiments or subculture were frozen at 1×10<sup>6</sup> cells/ml in GM plus dimethyl sulphoxide (DMSO; 10%). The cells were transferred to an isopropyl alcohol freezing chamber (Nalgene, NY, USA) and placed at -80°C to freeze the samples at a rate of 1°C/min. Following 24h, the cells were transferred and stored in a Statebourne cryogenics liquid nitrogen storage tank.

### 2.2.2. Differentiation of myoblasts

At confluence and following two washes with PBS, myoblasts were induced to differentiate by replacing GM with DM. After 48 hours (murine myoblasts) and (human cells), myotube formation became evident and progressed over the following 24-48 hours. Morphological, biochemical, flow cytometric and protein analyses were performed over a relevant time course as detailed below.

### 2.2.3. Cell counting by trypan blue exclusion

Cell counting was performed using a Neubauer haemocytometer (Figure.2.1). Data derived from this enabled the cell number per ml of suspension to be calculated. The proportion of dead cells was determined using the cell viability stain, trypan blue, which penetrates membranes of non-viable cells only. A mixture of cells: trypan blue (1:1) were counted in four grids (Figure.2.2) with each individual counting area of the haemocytometer representing a total volume of 0.1 mm<sup>3</sup>. The cell number per ml was calculated using the following calculation:

$$\text{Cells per ml} = \text{Average cell count in 4 grids} / 4 \times \text{dilution factor} \times$$

#### Equation 2.1. Calculation of viable and dead cell number following haemocytometer counting

Total cell number is calculated by multiplying the cells per ml with the total volume of the cell suspension from which the cells sample was removed.

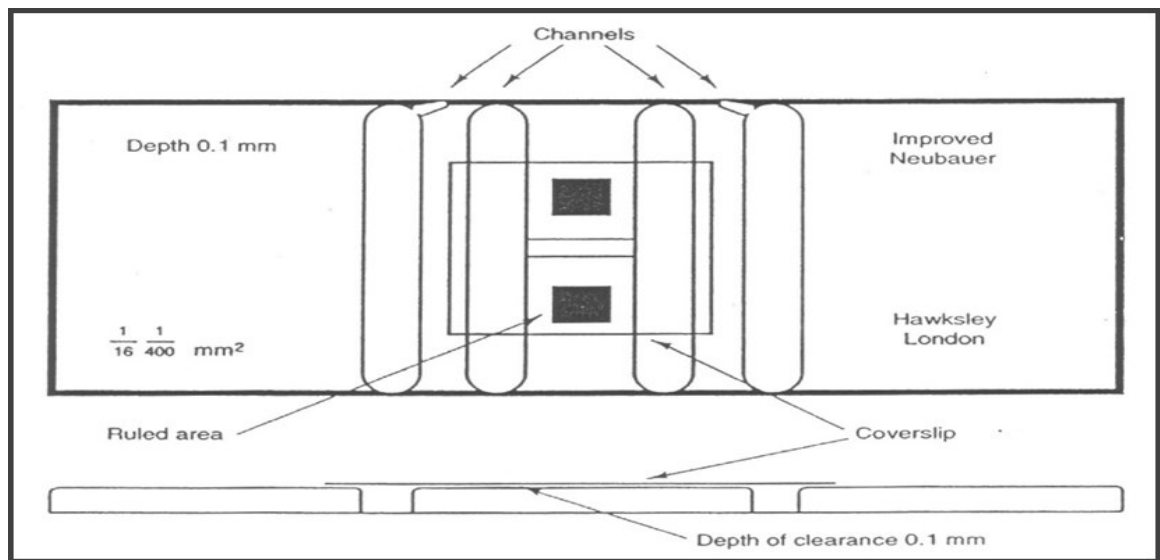


Figure 2.1. Neubauer haemocytometer (Figure taken from [www.sigmaaldrich.com](http://www.sigmaaldrich.com)).

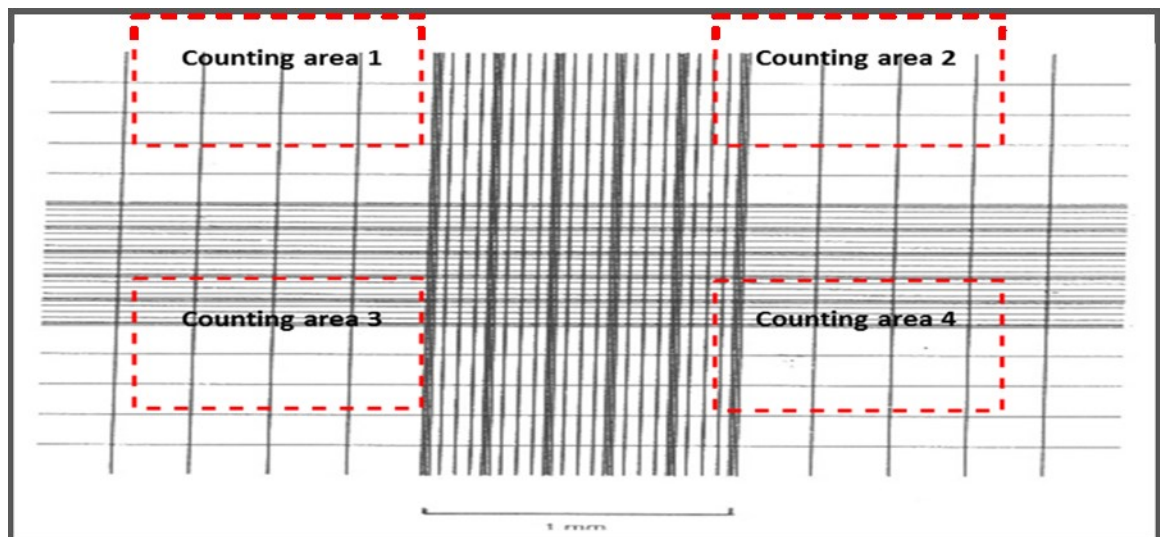


Figure.2.2. Haemocytometer counting grid (Figure taken from ([www.sigmaaldrich.com](http://www.sigmaaldrich.com))).

#### **2.2.4. Reconstitution of cytokines**

Recombinant human TNF- $\alpha$  (Life Technologies, UK) was reconstituted in 100  $\mu$ l sterile filtered water to produce a stock solution of 100  $\mu$ g/ml. Recombinant human IGF-I (Life Technologies, UK) was reconstituted in 200  $\mu$ l to produce a stock solution of 100  $\mu$ g/ml. Recombinant human IL-6 (Life Technologies, UK) was reconstituted in 50  $\mu$ l of acetic acid to produce a stock of 100  $\mu$ g/ml. All stocks were stored at -20°C until required.

##### **2.2.4.1. Culture with human recombinant cytokines**

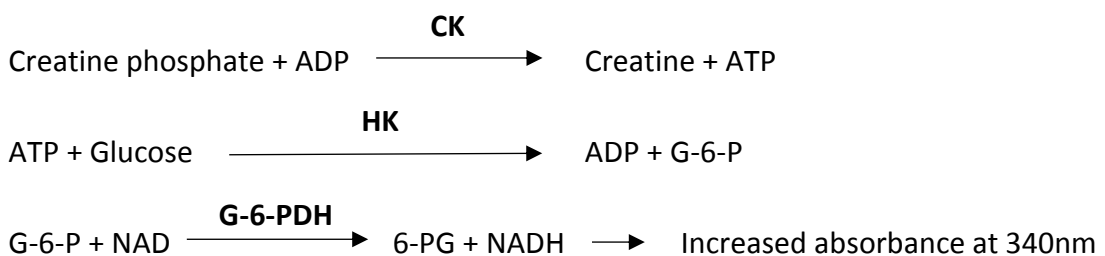
For experimentation, myoblasts were plated in duplicate in gelatin-coated plates at densities of: 60,000 cells per well (6-well plate) for mouse myoblasts and 100,000 cells per well (12-well plate) for human cells. Following overnight incubation, GM was aspirated and replaced with DM in the absence or presence of TNF- $\alpha$  (10 or 20 ng/ml), IL-6 (10 or 20 ng/ml) or IGF-I (30 or 100 ng/ml). Time courses of harvesting were performed as appropriate.

##### **2.2.5. Cell lysis for creatine kinase (CK) and total protein assays**

Following incubation for relevant times, cells were washed twice in PBS and lysed in Tris/MES Triton (50 mM Tris-MES, pH 7.8, 1% Triton X-100). Cells were lysed at room temperature for 10 minutes prior to storage at -20°C. Protein and CK assays were all completed within 2 weeks of the sample being obtained.

### 2.2.5.1. Creatine kinase assay

Creatine kinase (CK) activity can be used as a biochemical marker of the progression of *in vitro* muscle cell differentiation. In the creatine kinase assay, endogenously derived CK catalyses the reaction between creatine phosphate and adenosine diphosphate (ADP), forming creatine and adenosine triphosphate (ATP). Within this assay, the ATP formed is utilised to phosphorylate glucose, producing glucose-6-phosphate (G-6-P) in the presence of hexokinase (HK). Subsequently, G-6-P is oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NAD). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH). During the oxidation, an equimolar amount of NAD is reduced to NADH increasing the absorbance at 340nm. The rate of change of absorbance is directly proportional to endogenous CK activity.



Equation 2.2. Enzyme reactions involved in the creatine kinase (CK) assay (Smith *et al.*, 1985)

### 2.2.5.2. Creatine kinase assay procedure

Cells were differentiated for the relevant time periods prior to washing and harvesting as detailed above. The CK assay was performed at room temperature as follows: 4 µl of sample was added to 200 µl CK reagent in duplicate in a 96-well UV transparent flat-bottom plate. The contents were mixed and incubated for 3 minutes prior to serial reading (every minute for up to 20 minutes) at 340 nm using an ELx800 microplate reader (Biotek, USA). Initial and final readings were used to calculate the change in absorbance per minute ( $\Delta A/\text{min}$ ). CK activity (U/l) was calculated using the following equation:

$$\Delta A/\text{min} = ((\text{Final A} - \text{Initial A}) \div (\text{Final Reading Time} - \text{Initial Reading time (mins)}))$$

CK activity was then determined using the following calculation:

$$\text{CK (U/l)} = (\Delta A/\text{min} \times \text{TV} \times 1000) \div (6.22 \times \text{SV})$$

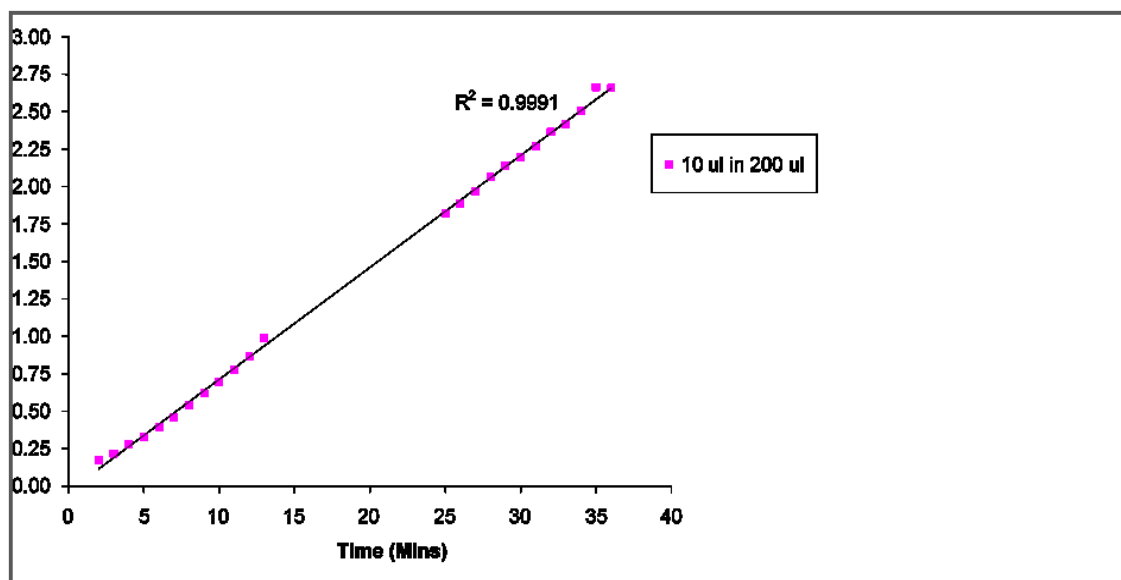
Where:

$\Delta A/\text{min}$	=	Change in absorbance per minute at 340 nm
TV	=	Total volume (ml)
SV	=	Sample volume (ml)
6.22	=	Millimolar absorptivity of NADH at 340 nm
1000	=	Conversion of Units per ml to Units per litre.

Therefore using 10 µl of sample and 200 µl of reagent the following equation applies:

$$\text{CK (U/l)} = (\Delta A/\text{min} \times 0.21 \times 1000) \div (6.22 \times 0.01)$$

**Equation 2.3. Calculation of creatine kinase activity(CK)**



**Figure 2.3. Linear Relationship of a single samples absorbance (y axis) per minute at 340 nm over 34 minutes**  
 There is good correlation observed ( $r = 0.99$ ) readings taken at 2-13 minutes (early) and 25 to 36 minutes (late) suggesting readings taken at an early time point are a good.

#### 2.2.5.2. Estimation of protein concentration

Bicinchoninic Acid™ (BCA) is used to determine the protein concentration in the cell lysates. Briefly, the assay detects  $\text{Cu}^+$ , which is formed when  $\text{Cu}^{2+}$  is reduced by protein in an alkaline environment (Smith *et al.*, 1985). The purple-coloured reaction product is formed by the chelation of two molecules of BCA with one  $\text{Cu}^+$ . This water-soluble complex exhibits a strong absorbance between 540 and 590 nm that increases linearly with increasing protein concentrations. Protein concentrations were determined with reference to Bovine Serum Albumin (BSA) standards of known concentration that were assayed alongside the cell lysates.

### **2.2.7. Immunocytochemical analysis**

Cell monolayers were washed in PBS, fixed in 3.7% formaldehyde, and incubated at room temperature for 10 minutes. The fixative was aspirated and the wells washed 3 times for 3 mins with PBS to remove excess paraformaldehyde prior to storage at 4°C for up to one week. Cells were washed briefly three times with PBS before being permeabilised with 0.1% Triton X-100 for 10 minutes at room temperature. Following Triton removal, cells were washed with PBS before staining with Texas Red-X Phalloidin at a final dilution of 1:1000. AntiMyosin Heavy Chain (MyHC) Alexa Fluor\_488 was added at 4 µg/ml and for nuclear staining, 4', 6-diamidino-2-phenylindole (DAPI) was added at a final dilution of 1:5000. After a 30-min incubation, the treated cells were photographed using a Leica DMI 6000B imaging system.



## **Chapter 3**

### **Effect of inflammatory cytokines on old C2 and young C2C12 myoblast muscle cells *in vitro***

### 3.1. Introduction

Many chronic diseases and aging are associated with elevated systemic inflammation. Inflammatory cytokines, such as (TNF- $\alpha$ ) and interleukin-6 (IL-6) have a negative impact on whole body function (Sprague & Khalil, 2009; Holecek, 2012), including inducing a reduction in muscle mass which is associated with a poor prognosis. Understanding the mechanisms underpinning inflammation-induced muscle wasting in chronic diseases such as muscular dystrophy, cancer, AIDS, chronic infections, and inflammatory myopathies, aging may ultimately provide routes for therapeutic interventions (Costamagna *et al.*, 2015a). Ways in which cytokines can affect muscle-wasting is via their impact on myoblasts survival and proliferation (Yin *et al.*, 2013).

By contrast, several polypeptide growth factors, including (IGF-I) have been implicated as stimulators of myogenic survival, proliferation and differentiation (Velloso, 2008; Yin *et al.*, 2013). Indeed, in aged muscle, the reduction in protein synthesis that is evident, is associated with reduced IGF-I availability (Barbieri *et al.*, 2003). In association with altered systemic environments, it is also possible that changes within the muscle itself may attenuate the responses to growth factor and cytokines with aging.

The objectives of this research were to identify differences in the response to TNF- $\alpha$ , IL-6 and IGF-I between the C2 and C2C12 myoblasts. It was hypothesised that responses to IL-6 and TNF- $\alpha$  would be exacerbated, whereas those to IGF-I would be blunted in C2 vs. C2C12 cells. The overarching aim of such research is to reduce muscle wasting with age.

### 3.2. Methods

The methods for myoblast treatment are detailed in the general methods chapter. Briefly, C2 and C2C12 murine skeletal myoblasts were exposed to DM, TNF- $\alpha$  (2.5 ng/ml or 10 ng/ml), IGF-I (50 ng/ml or 100 ng/ml) and IL-6 (2.5 ng/ml or 10 ng/ml) for 48 and 72hrs. The morphological alterations in differentiation were confirmed using biochemical analyses.

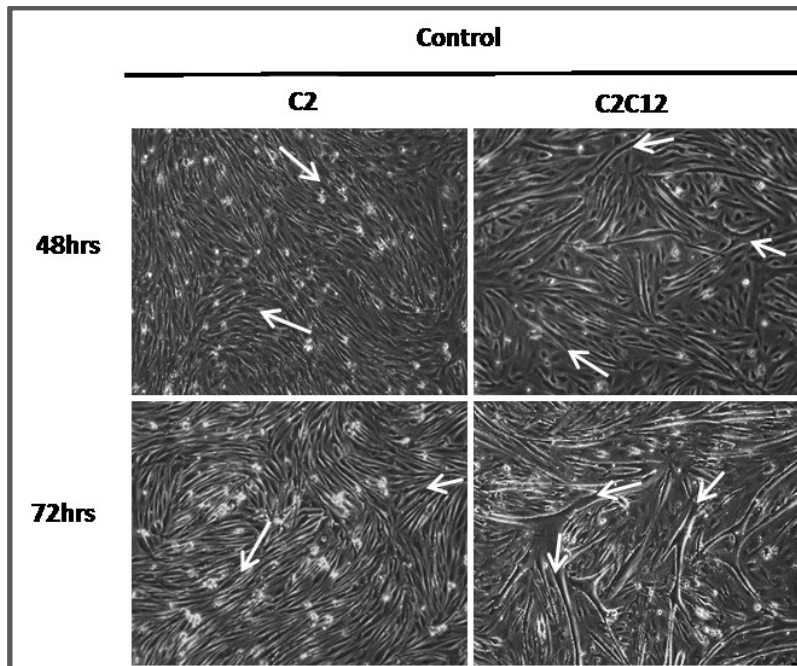
### **3.3. Statistics**

All experiments were repeated three times in duplicate, unless otherwise stated, and were analysed, using GraphPad Prism software version 5.0 (La Jolla, CA, USA). One-way ANOVA was used to compare the effects of all the experimental conditions followed by Bonferroni post hoc analysis. Results were presented as mean  $\pm$  standard deviation (SD). The significance was accepted as  $P \leq 0.05$ .

## 3.2. Results

### 3.2.1. Morphological differences in differentiation of young C2C12 compared with old C2 cells.

Both C2 and C2C12 cells showed evidence of fusion following 48 hours incubation under control conditions (Figure.3.1). Following 72 hours, however, the myotubes evident in the C2C12 cells are more extensive and abundant than those seen in the C2 cells, suggesting the C2C12 cells have greater myogenic potential than C2 cells.

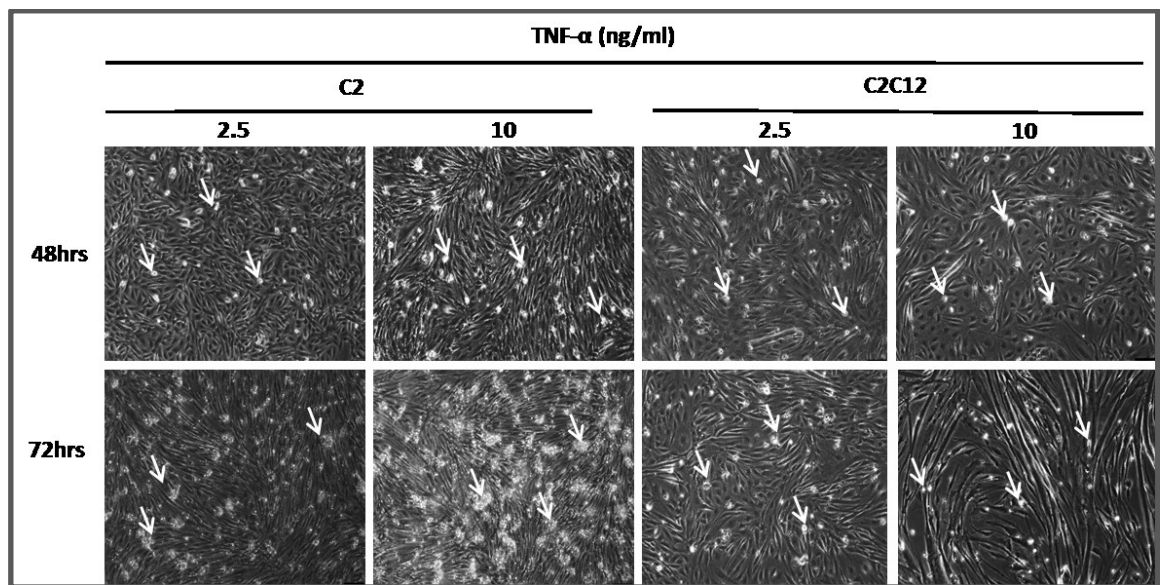


**Figure 3.1. Young C2C12 myoblasts cells showed more myotube formation compared to old C2 myoblasts cells.**

Old C2 do not differentiate as capably compared with young C2C12, which showed fully myoblasts fusion and differentiated myotube formation by 72hrs. Representative images of old C2 and young C2C12 mouse myoblasts cultured in differentiation medium (DM) for 48 and 72hrs. Arrows indicated the myotube formation in the old and young myoblasts. Images are representative of 3 experiments performed in duplicate. (10× magnification).

### 3.2.2. Impact of TNF- $\alpha$ on mouse skeletal muscle cells during differentiation

Experiments assessed dose responsive effects of 2.5 ng/ml or 10 ng/ml TNF- $\alpha$  on C2 and C2C12 myoblasts survival and differentiation at 48 and 72hrs. Photomicrographs illustrate a more potent impact of higher vs. lower dose TNF- $\alpha$  on the induction of cell death and inhibition of differentiation. This appears to be exacerbated in C2 vs. C2C12 myoblasts (Figure.3.2). Having determined that TNF- $\alpha$  is capable of reducing differentiation, most particularly in C2 vs. C2C12 cells, we next wished to determine the impact of IGF-I on cellular behaviour.

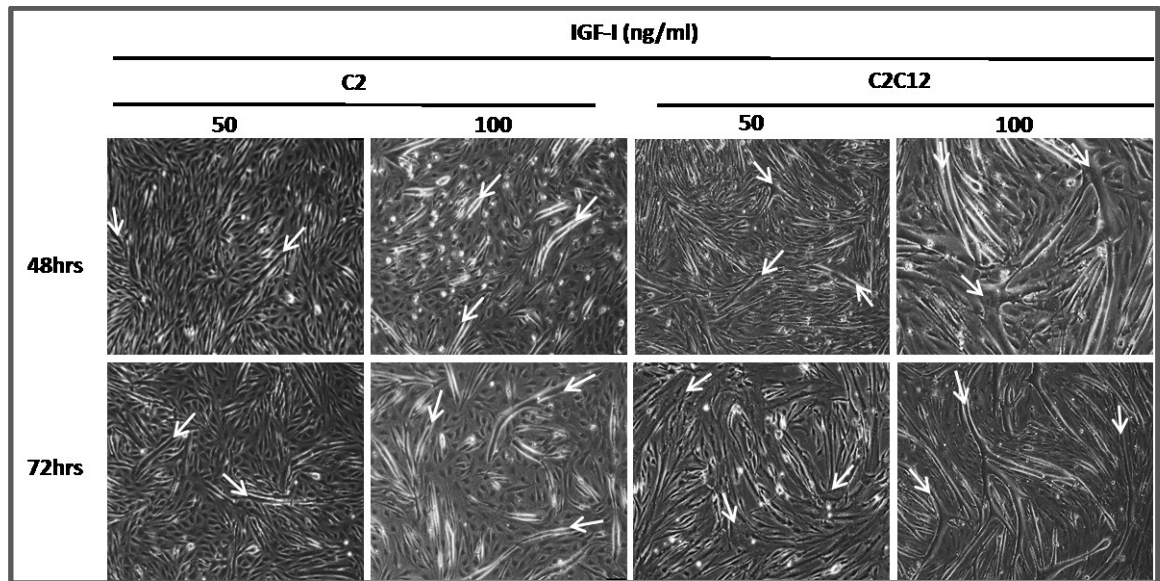


**Figure 3.2. Old C2 myoblasts cells induced more cell death compared to old C2C12 myoblasts cells.**

Images showed the lack of myotube formation and numerals of dead cells in C2 cells while C2C12 remained to differentiate with a few cell death. TNF- $\alpha$  inhibited myogenic differentiation in old C2 cells. Representative images of C2 and C2C12 myoblasts in the presence of TNF- $\alpha$  (2.5 ng/ml and 10 ng/ml) at 48 and 72 hrs. Arrows indicated the dead cells in young and old myoblasts. Images are representative of 3 experiments performed in duplicate. (10 $\times$  magnification).

### 3.2.3. Impact of IGF-I on mouse skeletal muscle during differentiation.

The impact of IGF-I (50 ng/ml and 100 ng/ml) on mouse skeletal myoblasts was investigated. In presence of IGF-I both myoblasts lines increased differentiation, however, this was greater in C2C12 vs. C2 cells, particularly at the higher dose (Figure.3.3). Having determined that C2 and C2C12 cells behave differentially under basal conditions and that their ability to survive and differentiate is diminished in the presence of both TNF- $\alpha$  and stimulated by IGF-I, we next wished to investigate the impact of IL-6 on myoblasts differentiation.

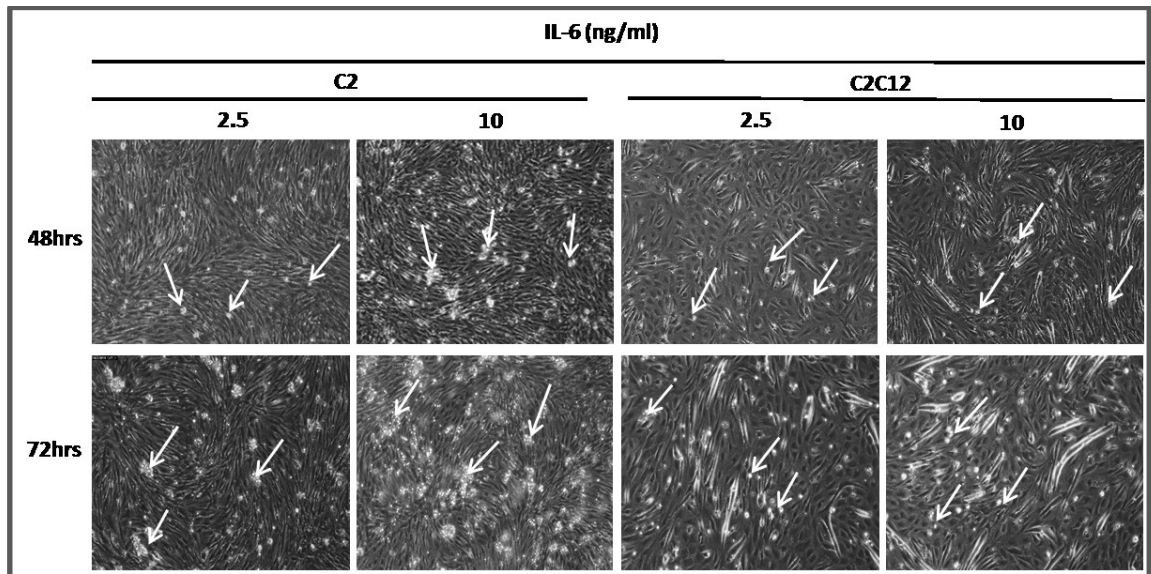


**Figure 3.3. Young C2C12 myoblasts IGF-I induced more myotube formation compared to old C2 myoblasts.**

C2C12 cells treated with high IGF-I dose induced number of myotube formation and more myoblast fusion compared to C2 cells, specifically at 72hss. IGF-I induced myogenic differentiation in young C2C12 cells. Representative images of C2 and C2C12 myoblasts in the presence of IGF-I (50 ng/ml and 100 ng/ml) at 48 and 72 hrs. Arrows indicated the myotube formation in the old and young myoblasts. Images are representative of 3 experiments performed in duplicate. (10 $\times$  magnification).

### 3.2.4. Impact of IL-6 on mouse skeletal muscle cells.

Cells were treated with IL-6 (2.5 and 10 ng/ml) for 48 or 72 hrs. IL-6 induced alteration on young (C2C12) and old (C2) myoblast cell morphology during differentiation. Morphological changes induced by IL6 were minimal at both times and in both cell lines (Figure. 3.4)



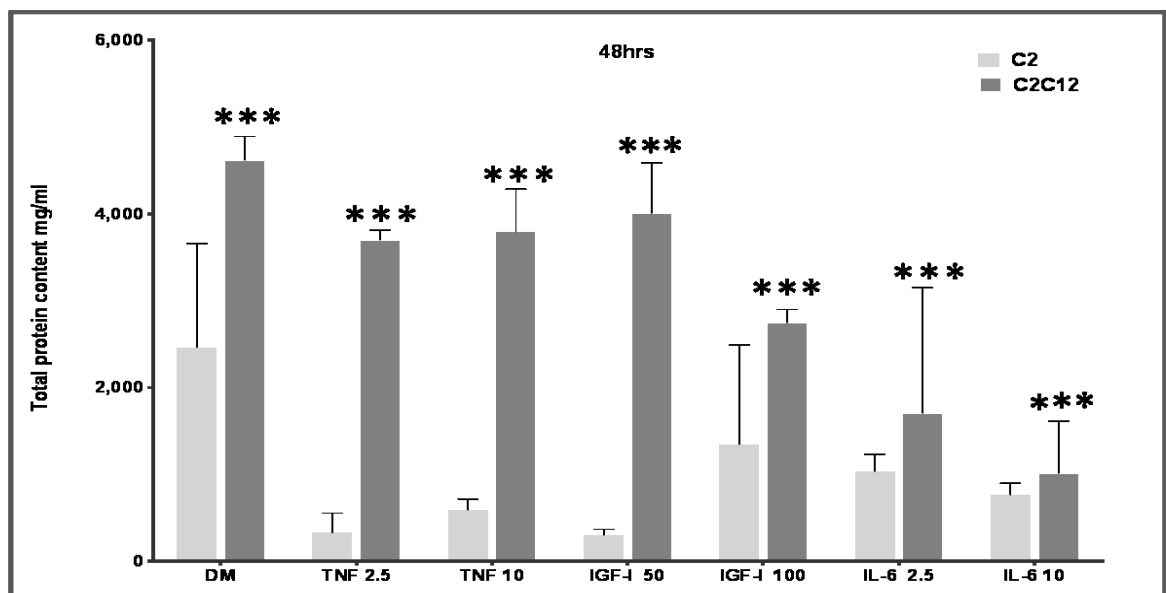
**Figure 3.4. Old C2 myoblasts cells induced more cell death and less myotube formation compared to young C2C12 myoblasts cells.**

C2C12 cells remained to differentiate (myotube formation) with small number of cell death particular with high dose. IL-6 were inhibited myogenic differentiation in old C2 cells. Representative images of C2 and C2C12 myoblasts in the presence of IL-6 (2.5 ng/ml and 10 ng/ml) at 48 and 72 hrs. Arrows indicated the dead cells in the old and young myoblasts. Images are representative of 3 experiments performed in duplicate. (10× magnification).

### 3.4.5 Biochemical differentiation of young and old murine myoblasts

Having determined that under control conditions, C2 cells appear to form smaller myotubes than C2C12 cells over a 72 hour period, are more prone to the negative impact of cytokines and resistant to the beneficial effects of IGF-I, we wished to confirm these findings at a biochemical level. Myoblasts were cultured as above and protein concentration and creatine kinase activity were assessed.

C2C12 cells displayed higher protein concentrations compared to C2 cells under all culture conditions (Figure.3.5). TNF- $\alpha$  elicited a reduction in protein concentration in C2 cells, which was not apparent in C2C12 cells. Surprisingly, IGF-I also caused a reduction in protein levels in C2 cells and at higher doses in C2C12 cells. Finally, IL-6, independent of dose, caused a reduction in protein levels in both cell lines.

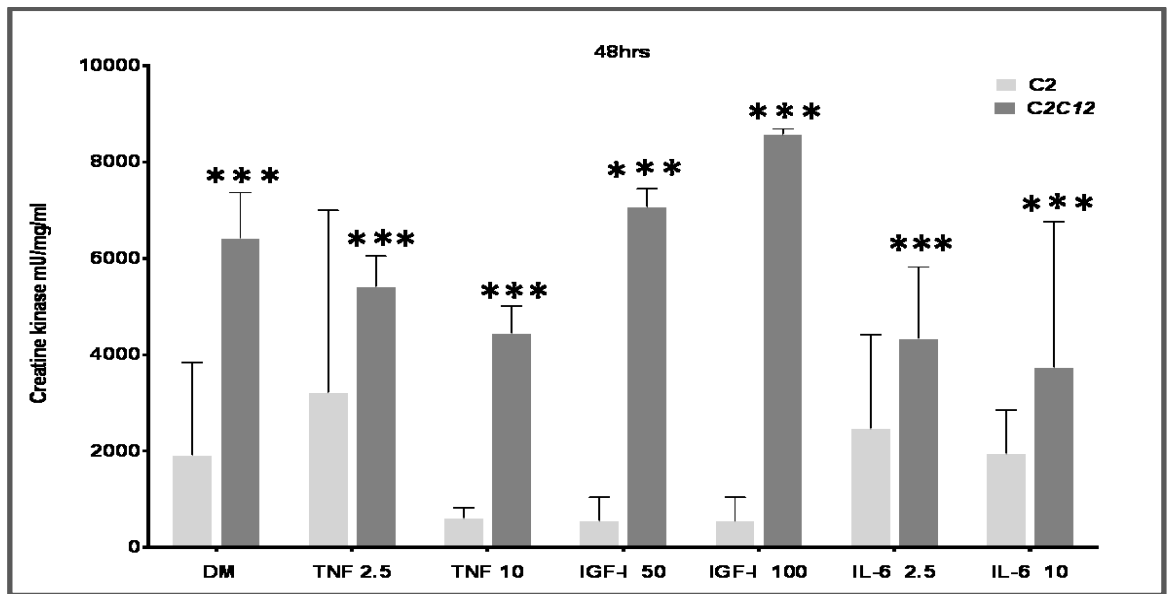


**Figure 3.5. Protein concentration of old C2 vs. young C2C12 mouse myoblasts in the presence and absence of TNF- $\alpha$ , IL-6 and IGF-I.**

In all condition, there was significant increased in total protein in young C2C12 cells vs. old C2 cells, in presence of TNF- $\alpha$ , IGF-I and IL-6. Data is taken from 3 experiments in duplicate. Graph depicts means  $\pm$  SD. \*\*\*P  $\leq$  0.001.



We next compared creatine kinase activity, a marker of biochemical differentiation, under the culture conditions described above and in both cell lines after 48hrs. C2 cells showed lower biochemical differentiation compared to C2C12 cells under all conditions. TNF- $\alpha$  induced a dose-dependent reduction in creatine kinase activity in both cell lines, but this was more pronounced in the C2 vs. the C2C12 cells. As anticipated, IGF-I elicited a dose-responsive increase in the C2C12 cells. By contrast, it was without beneficial effect in the C2 cells and indeed apparently suppressed CK activity in these cells, when compared with DM controls. Finally, IL-6 had little impact in both cell lines, compared with their own DM control.



**Figure 3.6. Creatine kinase (CK) activity of old C2 vs. young C2C12 mouse myoblasts in the presence and absence of TNF- $\alpha$ , IL-6 and IGF-I.**

Young C2C12 cells have higher CK activity than old C2 cells suggesting more differentiation. Data are taken from 3 experiments, in duplicate. Graph depicts means  $\pm$  SD. \*\*\* $P \leq 0.001$ .

### 3.3. Discussion

Within an aging population, a key question yet to be addressed is whether younger and older myoblasts behave differently to encounters with hypertrophic vs. atrophic agents. The models developed within this chapter provide a novel system with which to begin to address such questions. The main morphological and biochemical observation revealed by these studies is a better differentiation potential in the model of younger (C2C12) vs. older C2) myoblasts. When examining the impact of cytokines on myoblasts behaviour, it is immediately apparent that the C2 cells are more susceptible to the inhibitory effects of the higher dose TNF- $\alpha$  on differentiation, compared with the C2C12 cells. This effect is not evident with IL6 treatment, which at the doses investigated was with little impact on either cell line. Finally, while the C2C12 cells responded positively to IGF-I administration, with an increase in differentiation, this was not the case in the C2 cells and indeed these seemed to be negatively influenced by this growth factor at the doses tested. Overall, these data suggest, using a model of older and younger myoblasts, that younger cells respond as anticipated to both hypertrophic and atrophic agents. In accordance with expectations, the older cells are more responsive to the atrophic impact of TNF- $\alpha$ , but in contrast to hypotheses, IGF-I inhibited rather than stimulated myoblasts differentiation.

*In vivo* human studies suggest that a chronic, yet low level increase in systemic IL-6 may be responsible for the age-related decrease in muscle mass (Visser *et al.*, 2002; Barbieri *et al.*, 2003; Maggio *et al.*, 2006; Woods *et al.*, 2012). C2C12 cells do not support this observation. Although there is less fusion in the C2 vs. C2C12 cells under basal conditions, neither line displays an atrophic response to IL-6 administration. Therefore, the loss of muscle mass with age, may not be attributable to an increased systemic concentration of or sensitivity to IL-6 in old myoblasts.

Several studies of muscle loss with age and chronic disease suggest that TNF- $\alpha$  is a potent mediator of muscle wasting. Early *in vivo* rodent (Llovera *et al.*, 1993) and *in vitro* cell models (Stewart *et al.*, 2004) already supported this hypothesis. However, to date none have compared potentially exacerbated responses as a result of age. In the present study, we confirmed earlier observations of the detrimental impact of TNF- $\alpha$  on muscle cell differentiation. Here, we extend these findings to reveal that the differentiation of older C2 cells is impaired more extensively compared with the younger cell model. These data

complement studies by (Jejurikar *et al.*, 2006), who reported that older myoblasts in rodent models *in vivo* are more prone to the apoptotic impact of TNF- $\alpha$  vs. younger cells.

Finally, we also compared the impact of IGF-I on myoblasts behaviour. There are extensive data in the literature reporting the important pro-hypertrophic role of IGF-I in cells and in rodents e.g. (Stewart & Rotwein, 1996). Furthermore, there are also human association studies to suggest a role for IGF-I in hypertrophy in younger vs. older adults (Sharples *et al.*, 2015). Here, we report a divergence from these reported findings. In contrast to expectations, the C2C12 cells responded positively to the addition of exogenous IGF-I, however, the C2 cells appeared to atrophy. Previously we have reported that in the presence of low dose TNF- $\alpha$ , the co-incubation with IGF-I elicits a potent atrophic, rather than a hypertrophic response (Saini *et al.*, 2009). It is possible, although unlikely, that under the present culture conditions, exogenous IGF-I interacts with endogenously produced TNF- $\alpha$  (Greiwe *et al.*, 2001) by the older cells and that the combined impact is an induction of atrophy. It is important to note that this is a hypothesis to be tested, for which we have no current evidence.

### **3.4. Conclusion**

In this chapter, we report the comparison of parental and daughter cells under basal and stimulated conditions to ascertain the capability of developing a commercially available muscle cell model of age- and disease-related wasting. We establish that the older C2 cells are less able to hypertrophy and, as hypothesised, that the induction of atrophy with exogenous TNF- $\alpha$  is greater vs. C2C12 cells. In contrast to expectations, IL-6 had little impact on either cell line under the conditions of culture, whereas IGF-I elicited a hypertrophic effect in younger and an atrophic effect in older cells. This does indeed provide a useful model for further exploration of the ageing muscle cell process.

Critically, however, these studies have been undertaken in murine myoblasts with C2 cells being designated as older and C2C12 as younger. They are not of human origin and not from old vs. young humans. Therefore, in the next chapter we assess the response of old and young primary human skeletal muscle cell.

## **Chapter 4**

### **Effects of inflammatory cytokines on the differentiation of young and old human myoblasts *in vitro***

#### 4.1.1. Introduction

Age-related muscle wasting (sarcopenia) is a significant contributor to the loss of physiological and functional capacity in old age (Narici & Maffulli, 2010; Seene & Kaasik, 2012; Kalyani *et al.*, 2014) and has been strongly linked with morbidity and mortality (Kalyani *et al.*, 2014; Bonaldo & Sandri, 2013; Passey *et al.*, 2016). The loss of muscle mass occurs at a rate of 1-2% per year after the age of 50 years in humans (Kalyani *et al.*, 2014) and contributes to the majority of the age-related loss of muscle strength and therefore functional ability and metabolic performance of the older person (Bogdanis, 2012). More than 50% of the population older than 80 years suffer from sarcopenia, a condition recognised as a geriatric syndrome and a serious clinical disorder (Cruz-Jentoft *et al.*, 2010; Kalyani *et al.*, 2014; S. Ali & Garcia, 2014).

While some protein mobilisation from muscle protein is beneficial in acute trauma and disease, slight but persistent mobilisation of muscle protein in chronic conditions may result over time in large protein deficits and muscle atrophy that can have significant implications for the patient, including impaired respiration, mobility and efficacy of potential treatment (Stewart *et al.*, 2004). Moreover, the atrophied muscle may be more susceptible to injury during exercise and even during daily life activities (English & Paddon-Jones, 2010; Bogdanis, 2012). To repair such damage, satellite cells are required (Yin *et al.*, 2013).

Skeletal muscle has a notable ability to repair damage (Juhas & Bursac, 2013; Yin *et al.*, 2013). As skeletal muscle stem cells, satellite cells play an indispensable role in this process (Yin *et al.*, 2013). The self-renewing proliferation of satellite cells not only maintains the stem cell population but also provides numerous myogenic cells, which proliferate, differentiate, fuse, and lead to repair of damaged fibres or even new myofibre formation and reconstitution of a functional contractile apparatus (Yin *et al.*, 2013).

The ability of these cells to respond to growth and differentiation signals may be dependent on the local growth factor/ cytokine environment (Yablonka-Reuveni *et al.*, 1999). Indeed, cytokines play a crucial role in muscle regeneration by either stimulating or inhibiting proliferation or differentiation of myoblasts (Karalaki *et al.*, 2009). Prolonged elevations of proinflammatory cytokines (e.g. TNF- $\alpha$ ) and decreased growth factors, such as IGF-I, are associated with muscle wasting during aging and in several cachectic diseases e.g. AIDS,

rheumatoid arthritis and cancer (Saini *et al.*, 2010; Suzuki *et al.*, 2013). In line with this, in rodent models, TNF- $\alpha$  administration can induce a cachetic state (Stewart *et al.*, 2004) while in murine C2 and C2C12 skeletal muscle cell lines, TNF- $\alpha$  can inhibit myoblasts differentiation and lead to cell death (Stewart *et al.*, 2004). In the context of the present work it is important to note that circulating and local levels in skeletal muscle of TNF- $\alpha$  are increased in aging (Pedersen *et al.*, 2003).

It is not surprising that recent research has focused on the role of the interaction of growth factors and cytokines in the age-related muscle wasting. However, the majority of such *in vitro* studies have been carried out using immortalised rat and mouse muscle cell lines. While these studies have confirmed the importance of cytokines and IGF-I for myoblasts survival, differentiation and death, no studies have as yet investigated this in primary human myoblasts.

Therefore, the aim of the study in this chapter is to assess the impact of IL-6 and TNF- $\alpha$  on human skeletal muscle myoblasts survival, differentiation and death. It is hypothesised that TNF- $\alpha$  and, potentially IL-6, will cause impaired proliferation and differentiation and increased apoptosis that will be rescued using anabolic stimuli (IGF-I). To test these hypotheses, we compared (1) differentiation (protein, creatine kinase and myotube formation) of myoblasts obtained from young and old people and (2) the impact of cytokines with or without concomitant IGF-I supplementation.

#### **4.1.2. Methods and materials**

##### **4.1.2.1. Cell culture**

Young and old human skeletal myoblasts donated by the Butler-Browne laboratory (Bortoli *et al.*, 2003) were grown in T75 flasks in a humidified 5% CO<sub>2</sub> atmosphere at 37°C in growth medium (GM), until 90-100 % confluence was reached. Cells were incubated in presence and absence of TNF- $\alpha$  (10 ng/ml or 20 ng/ml), IL-6 (2.5 ng/ml or 20 ng/ml) and IGF-I (50 ng/ml or 100 ng/ml). For detailed methods of culture, cell isolation, histology and analyses, please see chapter 2.

##### **4.1.3. Statistical analysis**

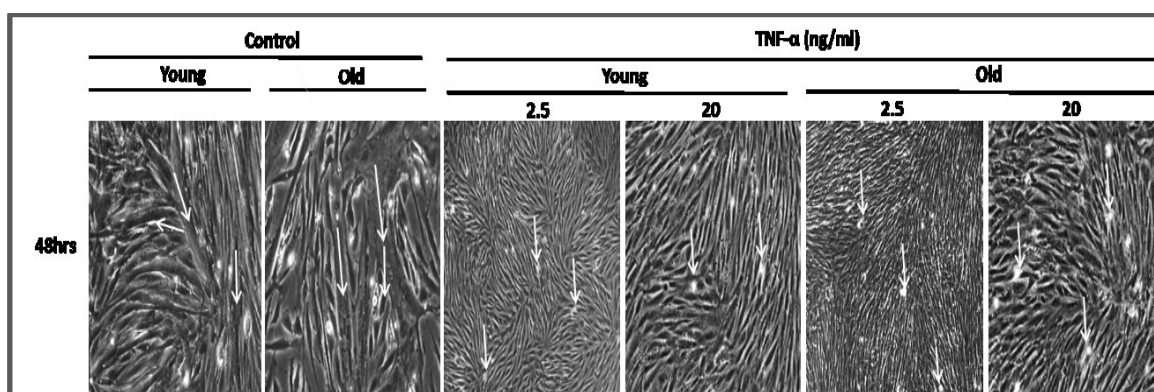
Statistical evaluation of data was performed using Student's *t*-tests. Results are presented as mean  $\pm$  standard deviation (SD). Differences are considered significant at  $P \leq 0.05$ .



## 4.2 Results

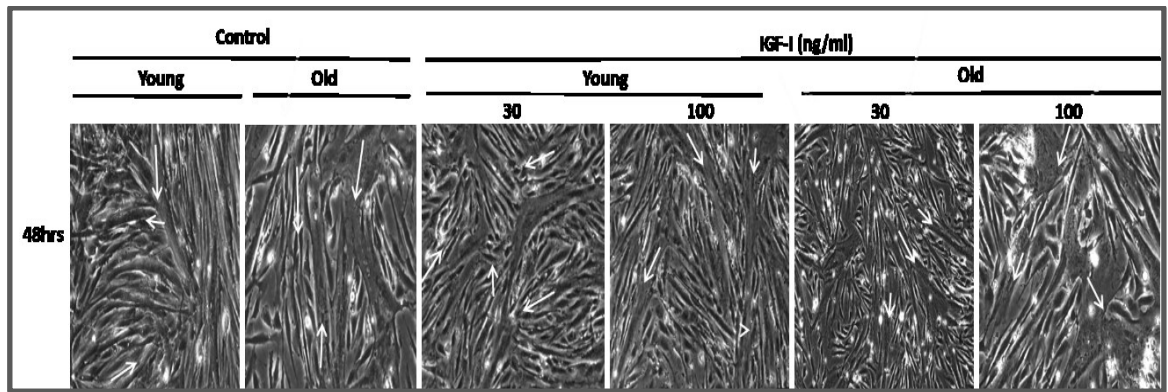
### 4.2.1. Morphological differentiation of young and old human myoblasts

Following transfer to differentiation medium, both young and old myoblasts were able to form large myotubes by 48hrs (Figure.4.1). Exogenous addition of TNF- $\alpha$  inhibited differentiation of both young and old myoblasts irrespective of dose (Figure.4.1). In the presence of the low dose of IGF-I, myoblasts fusion appeared to be impaired in both young and old human cells. At the higher dose of IGF-I there was little impact on fusion in young cells compared to baseline, but a small facilitation of fusion in old cells (Figure.4.2). Finally, addition of IL-6 to the human myoblasts appeared to be without significant impact on fusion potential in both young and old myoblasts, irrespective of dose (Figure.4.3).



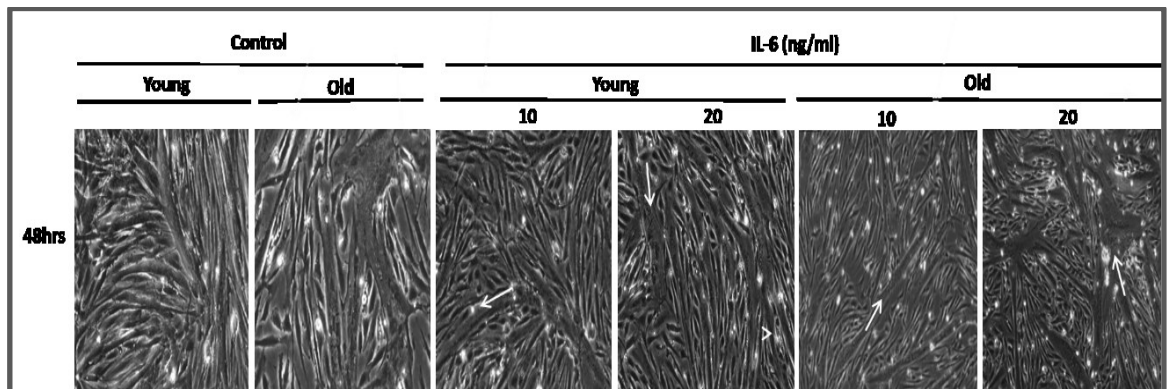
**Figure 0.1. TNF- $\alpha$  block differentiation and induced less myotube formation in both young and old myoblasts cells.**

Morphological images of young and old human myoblasts were induced to differentiate in the absence or presence of TNF- $\alpha$  (10 ng/ml and 20 ng/ml) at 48 hrs. Representative images show the lack of myotube formation and presence of numeral of cell dead cells in old and young cells, cell death was marked at the high dose of TNF- $\alpha$ . The white arrows showed dead cells. Images are representative of 3 experiments performed in duplicate. (10 $\times$  magnification).



**Figure 0.2. IGF-I induced myoblasts fusion and myotube formation in young and old myoblasts cells.**

Representative images of young and old human myoblasts in the absence or presence of IGF-I (30 ng/ml and 100 ng/ml) at 48 hrs. Images were showed differentiation and fusion of myoblast induced by IGF-I. Also Images were showed fully differentiated myotube in presence of numeral of cell dead in old and young myoblasts cells , especially with high IGF-I dose. The white arrows show the myotube formation in young and old myoblasts. Images are representative of 3 experiments performed in duplicate. (10× magnification).

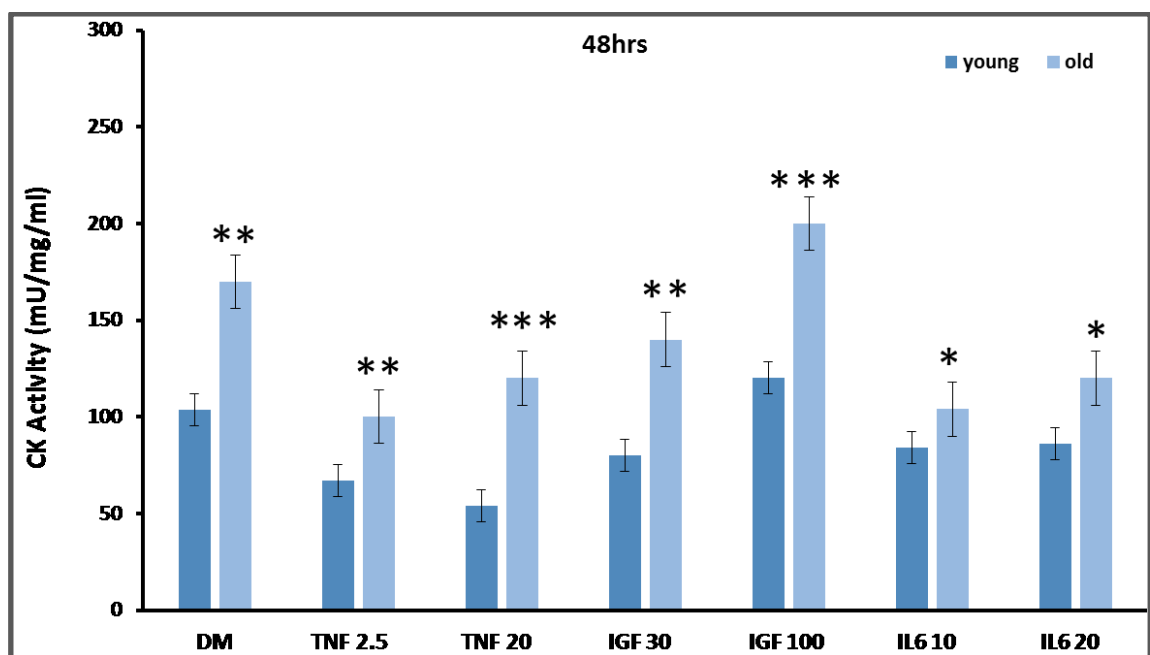


**Figure 0.3. IL-6 induced myotube formation in young and old human myoblasts cells with little number of cell dead.**

Representative images of young and old human myoblasts in the absence or presence of IL-6 (10 ng/ml and 20 ng/ml) at 48 hrs. Images showed the myotube formation and myoblasts fusion in presence of numeral of cell dead in old and young myoblasts cells. IL-6 induced differentiation and fusion in old and young myoblast cells. The white arrows show myotube formation and dead cells. Images are representative of 3 experiments performed in duplicate. (10× magnification).

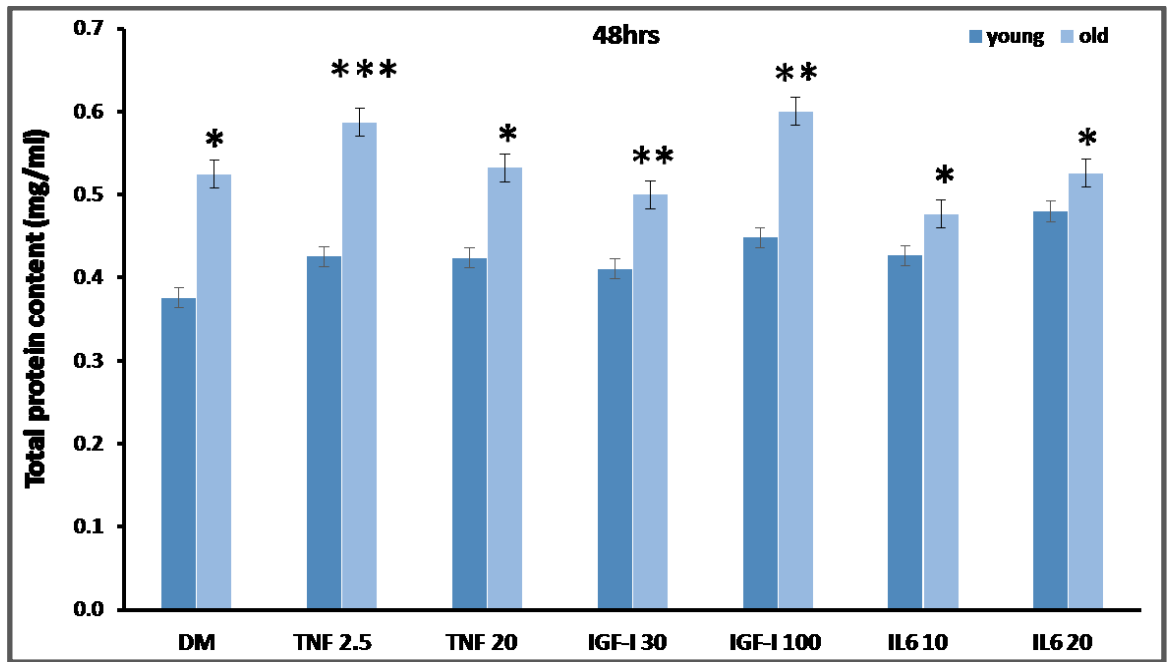
## 4.2. Biochemical differentiation of young and old human myoblasts

Following 48hrs of incubation in differentiation medium, protein levels were significantly higher in old vs. young myoblasts, irrespective of culture conditions (Figure.4.4). The creatine kinase assay provided evidence to suggest that old myoblasts are better at biochemical differentiation compared with young human myoblasts, regardless of treatment conditions. Furthermore, TNF- $\alpha$  induced a reduction in fusion in both old and young human myoblasts, irrespective of concentration (Figure.4.5), supporting the morphological findings. Again, in line with the morphological data, creatine kinase activity was reduced in the presence of the low dose of IGF-I in both young and old cells. The higher dose of IGF-I improved fusion in old and had little impact on fusion in young human myoblasts (Figure.4.5). In contrast to morphological data suggesting no impact on myoblasts fusion, the biochemical data indicate a suppression of differentiation in response to IL-6, most marked in the older human cells (Figure.4.5).



**Figure 4.4 Creatine kinase (CK) activity of old vs. young human myoblasts at 48hrs in the absence or presence of TNF- $\alpha$ , IGF-I and IL-6.**

Basally (DM) and IGF-I, the old cells have higher CK activity than young cells. Old cells and young cells have reduced CK activity in TNF- $\alpha$  and IL-6 condition. Data are taken from two experiments. Mean value  $\pm$  SE (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ). Data are taken from two experiments. Mean value  $\pm$  SE (\* $P \leq 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ) statistically significant.

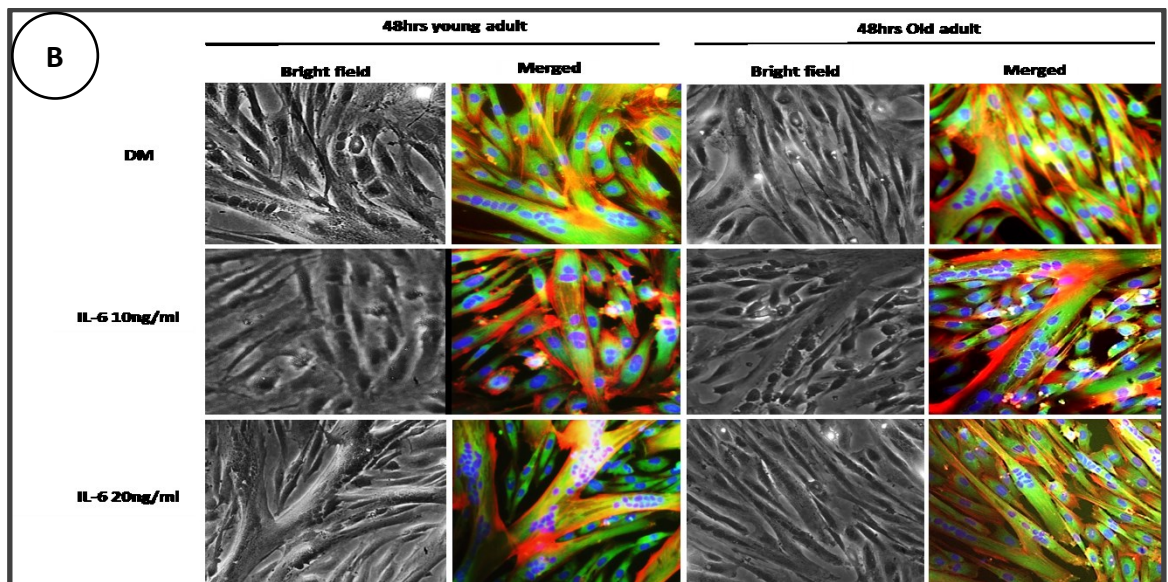
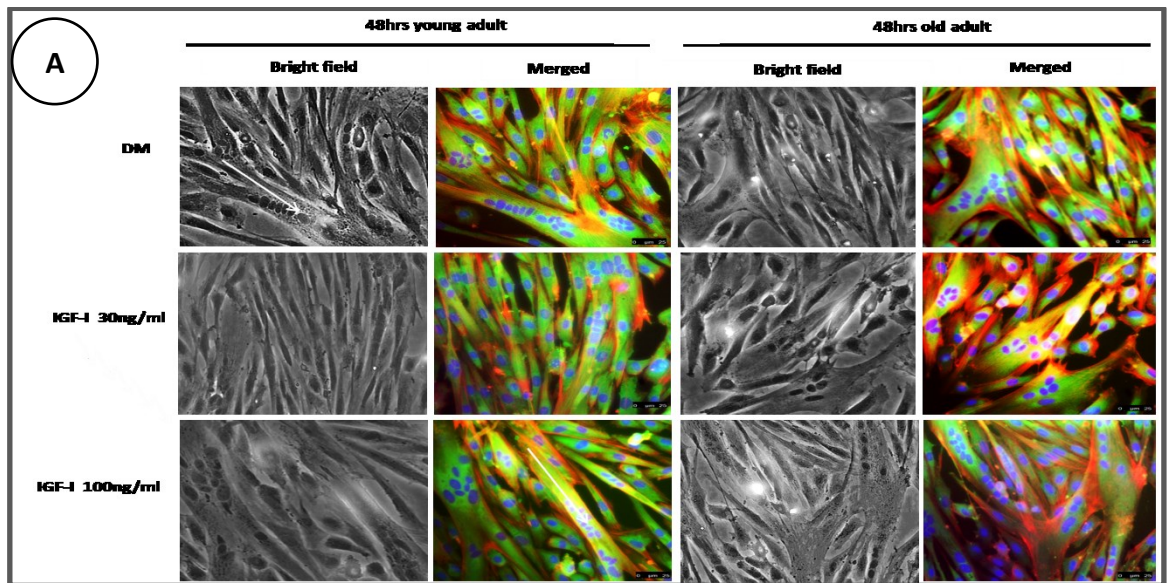


**Figure 0.4. Total Protein concentration of old vs. young human myoblasts cells at 48hrs .**

In all conditions, there was significant increase in total protein in old cells vs. young cells in presence of TNF- $\alpha$ , IGF-I and IL-6. Total Protein concentrations in old and old human myoblasts at 48hrs in the absence or presence of TNF- $\alpha$ , IGF-I and IL-6. Data are taken from two experiments, in duplicate. (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001) statistically significant.

#### **4.2.3. Immunocytochemical markers of differentiation in young and old human myoblasts**

The morphological and biochemical analyses revealed that TNF- $\alpha$  impaired fusion myoblasts. The derived data for IGF-I and IL-6 revealed some unexpected results, which warranted further investigation. To facilitate this process, cells were stained for actin and myosin (Figure.4.6). Easier visualisation of myotubes consolidated the prior findings, confirming reduced fusion in the presence of the low dose IGF-I in both young and old cells and improved fusion in old cells only at the higher dose (Figure.4.6). In line with the biochemical data, the immunocytochemical studies suggest that IL-6 suppressed differentiation (Figure.4.6).



**Figure 0.5. Immunofluorescent staining of young and old human myoblasts cells in the absence or presence of IGF-I and IL-6 at 48hrs.**

Figure ( A and B) young and old myoblasts cells were cultured in differentiation medium in presence or absence of A) IGF-I or B) IL-6 for 48 hrs to induce myotube formation. Cells were fixed and stained with anti MHC (green), Phalloidin (red) and DAPI (blue). Bright field images of differentiated old and young myoblast cultured in differentiation medium (DM) in presence and absence of IGF-I and IL-6. Merged images showed old and young myoblasts stained with anti MHC and Phalloidin to visualize myotubes. In addition, nuclei were visualized by DAPI staining. In A) it can be seen that high IGF-I dose improved the old myoblast fusion. Scale bar =25  $\mu$ m. Images were captured with a Leica DMI6000B microscope.

### 4.3. Discussion

Following the assessment of changes in murine models of age and inflammation, this chapter was devoted to extending murine models into old and young human myoblasts cultures. It was observed that old human myoblasts are capable of improved fusion compared with young human myoblasts. While TNF- $\alpha$  induced a reduction in fusion in both old and young human myoblasts, the response to IGF-I differed, in that the higher dose IGF-I improved fusion in old but had little impact on fusion in young human myoblasts. IL-6 suppressed differentiation, which was most marked in the older human cells.

In contrast to expectations and to extensive literature indicating age-related muscle wasting e.g.(Degens, 2007), we provide evidence to suggest that old muscle cells are in fact capable of efficient fusion. In contrast to the hypothesis that older muscles are less able to regenerate following injury compared with young muscle (Conboy et al., 2005; Carlson & Faulkner, 1989), the Harridge's group (Alsharidah *et al.*, 2013) provide convincing data to suggest that under basal conditions, mixed muscle-derived cell populations isolated from older or younger donors are indistinguishable in terms of myogenic potential. These apparently conflicting findings provide important insights into the role of the local. Systemic environment on myoblast behaviour. Our derived data extend the findings of Alsharidah *et al.* (2013), whereby a pure population of human myoblasts suggests even an improved capability of old muscle cell fusion.

To assess whether young and old myoblasts respond differently to the local environment, cells were exposed to equivalent culture conditions in the presence of relevant anabolic and catabolic agents. In line with expectations, TNF- $\alpha$  reduced myoblasts fusion (Stewart *et al.*, 2004), however, the degree of impairment was similar in both young and older myoblasts. The inhibition of fusion in the presence of IL-6 was greater in older than younger cells, perhaps underlying the age-related muscle wasting phenotype (Visser *et al.*, 2002). These findings do indicate that cytokines impact negatively on human-derived muscle cells and that cell age impacts on behaviour, although differing mechanisms of adaptation warrant investigation.

Wishing to determine the impact of a hypertrophic agent on human myoblasts adaptation, we investigated the response of the cells to IGF-I (e.g.( Stewart & Rotwein, 1996). Surprisingly, the lower dose of IGF-I resulted in a reduction, not an increase, in myoblasts

fusion in young and old myoblasts. By contrast, in old myoblasts, only, there was a hypertrophic response to the higher dose IGF-I. It has been reported that IGF-I acts to promote survival and proliferation as well as differentiation of myoblasts. One could therefore speculate, given the current data, that in younger cells IGF-I acts to facilitate survival as a dominant output, but in older cells, fusion is the preferential response. This is supported by data of Edstrom and Ulfhake suggesting the sarcopenia is not due to a lack of regenerative drive in old muscle (Edström & Ulfhake, 2005).

#### **4.4. Conclusion**

This chapter provides evidence of the development of a relevant human myoblasts model to study both fusion potential with age and the response of myoblasts to environmental stimuli. In addition to differences in basal fusion capability, where older myoblasts are as efficient as, or better than, younger myoblasts at forming myotubes, both young and old appear to respond differently to anabolic and catabolic stimuli.

## **Chapter 5**

### **General Discussion**



## 5.1. General Discussion

At the outset of this programme of research, it was hypothesised that murine and human myoblasts could be utilised interchangeably as models of muscle ageing and atrophy. Objectives were to investigate basal fusion potential of older and younger myoblasts and to examine the subsequent impact of growth factor/cytokine administration on myoblast differentiation. The aim was to provide a viable and relevant muscle cell model that can be used to reliably predict or screen the response to modulators of muscle mass. Table.5.1 summarises the main findings of the studies.

**Table 0.1. Summarises the main findings of the studies**

Summary of the differentiation responses of young and old human and murine myoblasts. In the control row, arrows indicate fusion differences between young and old cells and in the other rows, arrows indicate the response to the trigger administered, in the absence of an arrow, there was no change from baseline.

	Mouse Model		Human Model	
	C2C12(Young)	C2(Old)	Young	Old
Control	↑			↑
IGF-I low		↓	↓	
IGF-I high	↑	↓	↓	↑
TNF- $\alpha$	↓	↓ ↓	↓	↓
IL-6				↓

In the murine model, and in line with expectations, the higher dose IGF-I resulted in improved fusion in younger, but reduced fusion in older, myoblasts. By contrast and unexpectedly, in human myoblasts the opposite observation was made, where the higher dose of IGF-I resulted in reduced fusion in younger but increased fusion in older myoblasts. The improved fusion in both models was associated with enhanced fusion potential at the baseline, which should therefore be considered when choosing models of study. It is currently not known what drives altered basal fusion capability and the subsequent enhanced response to IGF-I. Therefore, the two models together provide an opportunity to further investigate this finding.

When assessing the impact of catabolic cytokines on myoblasts fusion, both models were consistent in greater negative responses elicited by older vs. younger myoblasts, despite differences in detail e.g. older murine myoblasts are more responsive to TNF- $\alpha$  and older human myoblasts to IL-6 administration. The impaired differentiation in response to the cytokines may underlie the muscle wasting evident in older age.

In progressing these models further, several questions arise relating to both growth factor and cytokine responses, however the most exciting questions which immediately arise are related to basal and IGF-I responses, as follows:

1. What defines the basal difference in fusion capacity within the two models?
2. Why do the two models differ in their age-related response to IGF-I?

To address these questions, molecular and intracellular signalling technologies need to be applied. If fundamental mechanisms can be determined and manipulated, they provide relevant information for pharmacological interventions in muscle wasting disorders.

## **5.2. Limitations**

Interactions between growth factors and cytokines, which are known to play an important role in muscle behaviour, have not been investigated. It is also noted that in any organism, there are significantly more factors that impact in concert than those which we have chosen to study here in isolation. However, as an advantage, the approach utilised here can define cleanly the role of an isolated stimulus on, in this case, myoblasts behaviour that can be used as a foundation for further study.

## **5.3. Conclusion**

The concluding observation of these studies is that the impact of aging on fusion capability differs between murine and human myoblasts. Part of the differential response to stimuli in these models may be related to the basal differences in fusion capacity. Therefore, care should be exerted when selecting the model of choice as the two cannot be used interchangeably. They do, however, potentially provide useful stand-alone models to investigate the mechanisms of action of a wide variety of stimuli that have an impact on muscle mass.

## 5.4. Future directions

On the basis of the findings of this research, there arise a number of options for future research including:

- Molecular analysis to ascertain difference between key genes involved in the proliferation and differentiation, including IGF-I, myogenic differentiation factor (MyoD, myogenin, Id3) and TNF- $\alpha$  in normal differentiation conditions.
- This thesis has shown that differentiation potential and the differentiation time course were different between cells from the young and old at different time courses. We would have next examined the different groups age at different time course.
- The work presented in this thesis has shown the impact of TNF- $\alpha$ , IL-6 and IGF-I on young and old skeletal myoblasts cells, which have effects on myogenic behaviour in a dose-dependent manner. The next step would be to study the signalling pathways involved to determine the interaction between the TNF- $\alpha$  and IGF-I or IL-6 and IGF-I, which affect skeletal muscle.
- Another potential consideration for future work would be to alter the method of protein analysis for western blot analysis; blots could be stripped and probed using a control such as actin or tubulin. These proteins are often used as a control because their levels are rarely changed by treatment and provide a method to verify that the same amount of sample protein has been loaded in each gel lane and transferred to the membrane.
- Obtaining muscle biopsies from elderly and young subjects would allow assessment of cellular and molecular differences.
- To further understand the mechanism behind the differences in differentiation and TNF- $\alpha$  induced apoptosis between young and old cell, an investigation of cytoplasmic and nuclear protein that may be involved could be undertaken.

## **Chapter 6**

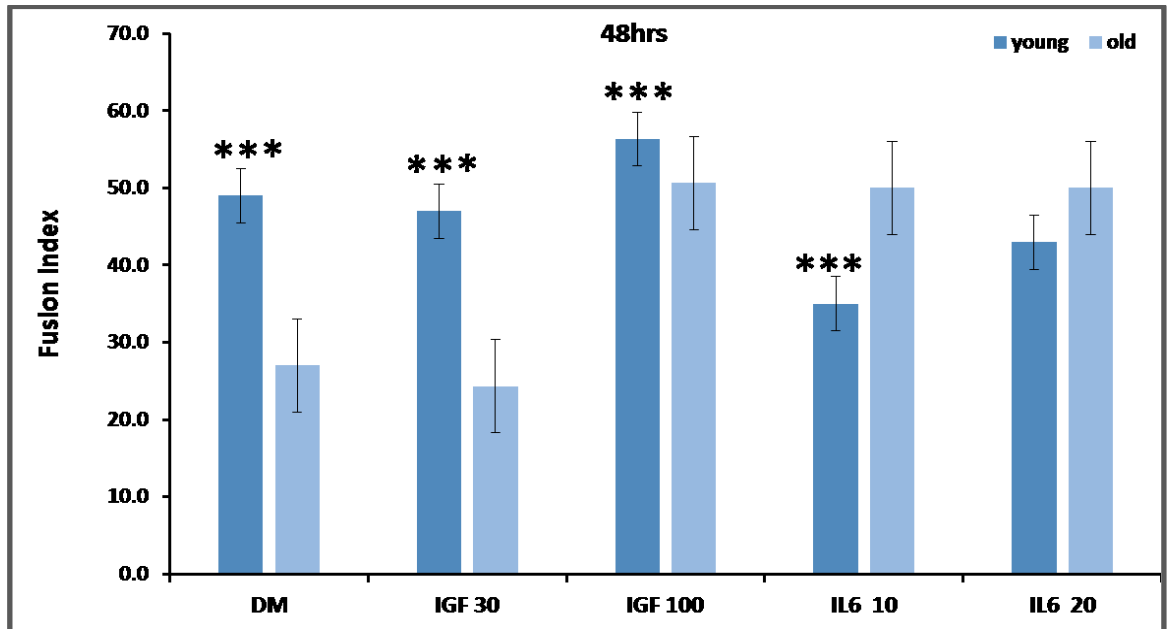
## **Appendices**

## 6.1. Web sites

<http://www.bdcompany.com/>

[www.sigmaaldrich.com](http://www.sigmaaldrich.com)

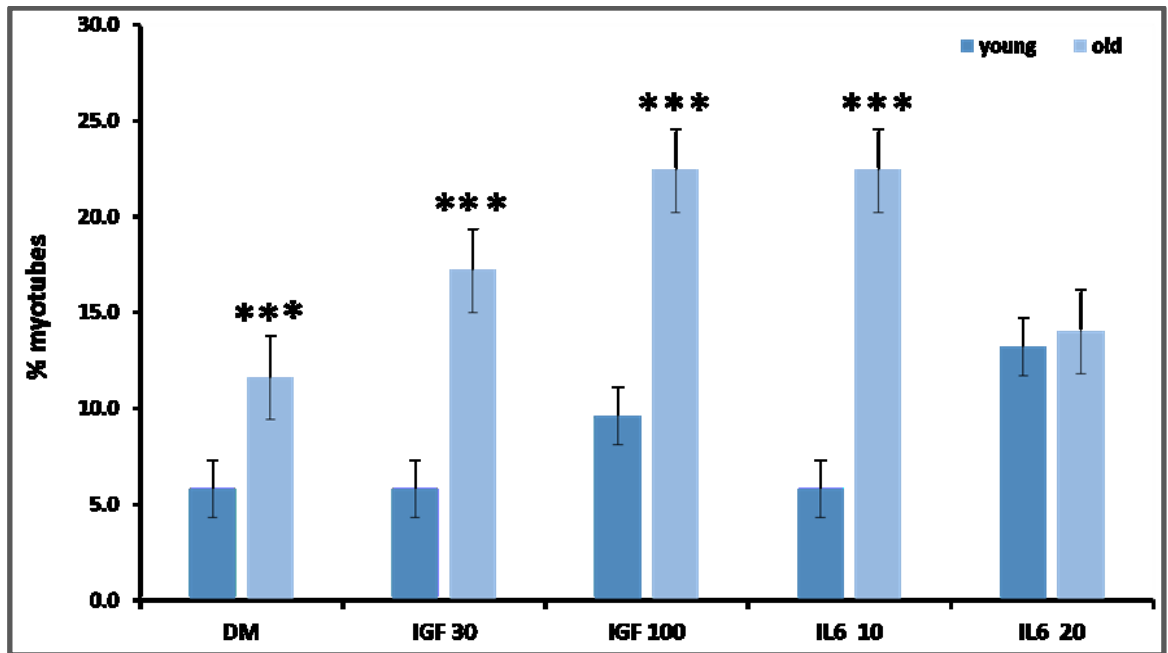
## 6.2 Appendix 1



**Figure 0.1. Total fusion index of old and young myoblast cells.**

Fusion index was measured in myotube differentiated in presence and absent IGF-I and IL-6 for 48hrs. Total fusion index analysis representing the number of nuclei in differentiated myotubes divided by total number of nuclei present in the observed field, with a myotube defined by at least 2 myonuclei. Nuclei were counted from random fields of each line at 5 in DM, IGF-I and IL-6 in a varying doses (determine by immunofluorescence staining) P value was determined with a t-test. Data presented as average  $\pm$ SD. \*\*\*  $p < 0.001$ .

### 6.3. Appendix 2



**Figure 0.2. The percentage of myotubes of young and old myoblasts cells.**

The percentage of myotubes in the cell cultures after administration of 30 ng/ml or 100 ng/ml IGF-I and 10 ng/ml or 20 ng/ml IL6. Effect of IL-6 and IGF-I stimulation on myotube formation at 48hrs of differentiation. The percentage of myotubes was higher with higher doses at 72hrs in old cells vs. young cells. P value was determined with a t test. Data presented as average  $\pm$ SD. \*\*\*  $p \leq 0.001$ .

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